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(54) Title: CELL DEATH PROTEIN

(57) Abstract

Disclosed is a protein, designated RIP, which contains a death domain at its carboxy terminus and a kinase domain at its amino terminus. RIP interacts with the Fas/APO-1 intracellular domain and the TNFR1 intracellular domain. When expressed in transformed host cells, recombinant RIP promotes apoptosis. Also disclosed are DNA molecules encoding RIP, anti-RIP antibodies, and screening methods for discovering inhibitors of RIP-dependent apoptosis.

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CELL DEATH PROTEIN

Field of the Invention

This invention relates to recombinant DNA,
proteins encoded by recombinant DNA, and screening
methods for discovery of compounds with pharmacological
activity.

Background of the Invention

Regulated (or "programmed") cell death is
essential for the orderly development of metazoan
organisms and is crucial to the proper functioning of the
immune system in higher vertebrates (Wyllie et al., Int.
Rev. Cytol. 68:251-306, 1980). The most common
morphologic expression of programmed cell death is
apoptosis, characterized by cell shrinkage, zeiosis, or
blebbing, of the plasma membrane, and nuclear collapse
and fragmentation of the nuclear chromatin, at
intranucleosomal sites, due to activation of an
endogenous nuclease (Clement et al., J. Exp. Med.
180:557, 1994). Apoptosis is involved in morphological
development, precise regulation of cell numbers, and as a
defense mechanism to remove unwanted, and potentially
dangerous cells, such as self-reactive lymphocytes, cells
infected by viruses, and tumor cells. See, e.g.,
Steller, Science 267:1445, 1995; Thompson, Science
267:1456, 1995. Diseases associated with increased
apoptosis include AIDS, neurodegenerative disorders
(e.g., Alzheimer's disease, Parkinson's disease,
amyotrophic lateral sclerosis, retinitis pigmentosa),
aplastic anemia, ischemic injury (e.g., myocardial
infarction, stroke, reperfusion injury), and toxin-
induced (e.g., alcohol induced) liver disease.

An important mediator of immunologically relevant
cell death is the Fas antigen/APO-1 (also known as CD95)
("Fas/APO-1"), originally identified as the target of

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monoclonal antibodies that could kill multiple cell types
(Trauth et al., Science 245:301, 1989; Yonehara et al.,
J. Exp. Med., 169:1747, 1989). Cloning of cDNA, followed
by sequence analysis (Itoh et al., Cell 66:233, 1991;
5 Watanabe-Fukanaga et al., J. Immunol. 148:1274, 1992a;
Oehm et al., J. Biol. Chem. 267:10709, 1992) showed
Fas/APO-1 to be a member of a family of transmembrane
receptors that includes the low affinity nerve growth
factor ("NGF") receptor, the tumor necrosis factor
10 receptors ("TNFR1," "TNFR2"), and a variety of immune
cell receptors including CD40, OX40, CD40, CD27, and 4-
1BB (see, Smith et al., Cell 76:959, 1994). In addition
to Fas/APO-1, several members of this family have been
shown to regulate or induce cell death, e.g., p55 TNFR
15 (TNFR1) Tartaglia et al., Proc. Natl. Acad. Sci. 88:9292,
1991; Tartaglia et al., Cell 73:213, 1993b) and p75 TNFR
(TNFR2) (Heller et al., Cell 70:47, 1992; Heller et al.,
Cell 73:216, 1993; Clement and Stamenkovic, J. Exp. Med.
180:557, 1994).

20   Disruption of Fas/APO-1 expression or function in
lymphoproliferation ("lpr") mutant mice leads to a
progressive lymphadenopathy and an autoimmune syndrome
resembling human systemic lupus erythematosis (Watanabe-
Fukunaga et al., Nature 356:314, 1992b). The residual
25 cytotoxic activity of T-cells derived from perforin
deficient mice is also dependent on the presence of at
least one wild type allele of the *lpr* locus (Kagi et al.,
Science 265:528, 1994; Kojima et al., Immunity 1:357,
1994; Lowin et al., Nature 370:650, 1994). Thus the
30 ability of Fas/APO-1 to induce cell death is important
for the maintenance of at least two immunologic processes
in vivo: peripheral tolerance to self (Singer and Abbas,
Immunity 1:365, 1994; Crispe, Immunity 1:347, 1994), and
calcium-independent T-cell cytotoxicity (Henkart,
35 Immunity 1:343, 1994).

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The mechanism by which Fas/APO-1 induces cell
death is unknown, but it requires multivalent cross-
linking of the receptor (Dhein et al., J. Immunol.
149:3166, 1992) and is facilitated by concurrent
5   inhibition of RNA or protein synthesis in some cell
types. Other factors have been reported to modulate
Fas/APO-1 activity (Klas et al., Int. Immunol. 5:625,
1993), and, under certain circumstances, Fas is capable
of signaling activation rather than death (Alderson et
10   al., J. Exp. Med. 178:2231, 1993). Anti-Fas antibodies
and TNF are both capable of signaling cell death *in vitro*
with similar kinetics (Yonehara et al., (supra); Itoh and
Nagata, J. Biol. Chem. 268:10932, 1993), and among
members of the NGF/TNF receptor family, Fas/APO-1 and
15   TNFR1 share the most significant cytoplasmic homology.

Fas/APO-1 contains sequences required for the cell
death response (Itoh and Nagata (supra) 1993). It has
been proposed that a "death domain" contained in a region
of similarity between Fas/APO-1 and TNFR1 is essential
20   for the initiation of apoptosis by both molecules,
perhaps through an interaction with other intracellular
proteins (Tartaglia et al., Cell 74:845, 1993).

Summary of the Invention

We have discovered a protein, designated RIP (for
25   Receptor Interacting Protein), which contains a death
domain at its carboxy terminus and a kinase domain at its
amino terminus. We have further discovered that RIP
interacts with the Fas/APO-1 intracellular domain.
Moreover, RIP overexpression in eukaryotic cells leads to
30   apoptosis. The ability of RIP to associate with the
Fas/APO-1 intracellular domain, and to promote apoptosis,
indicates that it is an important element in the signal
transduction machinery mediating programmed cell death.

Accordingly, the invention features an RIP,
35   comprising a death domain in its carboxy terminal region

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and a kinase domain in its amino terminal region, said
RIP being capable of interacting with cellular factors
such as the Fas/APO-1 intracellular domain, or the TNFR1
intracellular domain, and said RIP being capable of
5   promoting apoptosis in a eukaryotic cell. The invention
also features RIP fragments, e.g., the RIP death domain,
capable of interacting with one or more cellular factors
and capable of promoting apoptosis.
        The invention also features an isolated
10 recombinant DNA molecule encoding an RIP, or encoding a
fragment thereof (e.g., the RIP death domain) and a host
cell transformed with the recombinant DNA molecule. The
transformed host cell is used to express recombinant RIP.
        The invention also features anti-RIP antibodies,
15 which are useful as medical diagnostic reagents, as
affinity chromatography reagents for isolation of RIP,
and as analytical scale laboratory reagents for use in
research on the physiology and cell biology of apoptosis.
        The invention also features screening assays to
20 identify inhibitors of *in vivo* binding interactions
mediated by the RIP death domain. Isolated RIP,
recombinant RIP, or a binding domain thereof, is an
essential component of the screening assays of this
invention.
25   The invention also features screening assays to
identify inducers of RIP expression and resulting
apoptosis.
        The invention also features methods of producing,
for introduction into a patient undergoing gene therapy,
30 cells that can be induced to undergo apoptosis upon
administration of an exogenous agent to the patient.
        As used herein, the term "Act-Fas" means a
transcriptional activator fusion protein expressed by a
library isolate, wherein the activator is fused to a
35 Fas/APO-1 sequence.

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As used herein, the term "Act-RIP" means a transcriptional activator fusion protein expressed by a library isolate, wherein the activator is fused to an RIP sequence.

5   As used herein, the term "apoptosis" means non-necrotic cell death that takes place in metazoan animal cells following activation of an intrinsic cell suicide program. Apoptosis is a normal process in the development and homeostasis of metazoan animals.

10 Apoptosis involves characteristic morphological and biochemical changes, including cell shrinkage, zeiosis, or blebbing, of the plasma membrane, and nuclear collapse and fragmentation of the nuclear chromatin, at intranucleosomal sites, due to activation of an

15 endogenous nuclease.

As used herein, the term "death domain" of RIP means the 98 C-terminal amino acid residues of RIP, which display sequence relatedness to an approximately 90-amino acid residue C-terminal region in Fas/APO-1 and TNFR1.

20   As used herein, the term "interacting" means specifically associating as a result of 3-dimensional structure; specific non-covalent binding.

As used herein, the term "kinase domain" of RIP means the amino terminal region of RIP (i.e., from a few

25 residues after the N-terminal methionine residue to approximately residue number 300), which has sequence relatedness to known tyrosine kinases and serine/threonine kinases.

Other features and advantages of the invention

30 will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Fig. 1 is a graph summarizing quantitative β-galactosidase assay results. Three colonies from each

35 Act-RIP/LexA-fusion protein pair were used to inoculate a

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galactose-containing liquid culture. The β-galactosidase
activity of lysates prepared from each culture was
measured and normalized to the total protein
concentration of the lysate. 5 units (nmoles/min/mg
5 protein) represents the limit of detection of β-
galactosidase activity in this system.

Fig. 2 is a set of genetic maps comparing RIP
cDNAs. The inserts of clones isolated from a mouse
thymus cDNA library are symbolized as lines above a bar
10 diagram of the composite sequence, which depicts the
regions encoding the kinase domain (hatched box), the
death domain (black box), and a region of unknown
function predicted to have high α-helical content
(unshaded). Also shown is the region of human RIP that
15 was used as a probe to isolate murine cDNAs. The
original human RIP isolate encompassed sequences encoding
the death domain and about 100 residues upstream.

Fig. 3 is the deduced complete amino acid sequence
of murine RIP and a deduced partial human RIP amino acid
20 sequence.

(B) Deduced polypeptide sequence. The mouse
sequence consists of a contiguous open reading frame
proceeding from a translational initiation consensus.
The human sequence predicted from a cDNA fragment is
25 shown below the mouse; identical residues are indicated
by a dash, and gaps indicated by a period. The conserved
consensus sequences for casein kinase II (S-X-X-E) and
cAMP- or cGMP-dependent protein kinase (R-X-X-S) are
overlined.

30   Fig. 4 is the amino acid sequence of the RIP Death
Domain. Sequence alignment of the RIP C-terminus with
the death domains and carboxyl termini of Fas/APO-1 and
TNFR1. Gaps are indicated by dashes. Consensus residues
conserved in all six sequences are capitalized, whereas
35 positions at which a charge-conserved residue is found in

1/6 sequences are shown in lower case. Noteworthy charge
conservations are bolded. The regions denoted **A** and **B**
represent two portions of the Fas/APO-1 cytoplasmic
domain completely conserved between mouse and human.

Detailed Description

5   The present invention provides a novel recombinant
protein, designated RIP, and DNA encoding the protein.
RIP contains an amino-terminal kinase domain and a
carboxy-terminal death domain, through which RIP
10 associates with the Fas/APO-1 intracellular domain, and
to a lesser extent, the TNFR1 intracellular domain. RIP
appears to be an apoptosis-inducing nonreceptor kinase.
Accordingly, RIP appears to be an important element in
the signal transduction pathway mediating programmed cell
15 death.
       Various diseases, including AIDS,
neurodegenerative disorders (e.g., Alzheimer's disease,
Parkinson's disease, amyotrophic lateral sclerosis,
retinitis pigmentosa), aplastic anemia, ischemic injury
20 (e.g., myocardial infarction, stroke, reperfusion
injury), and toxin-induced (e.g., alcohol induced) liver
disease, involve abnormal increases in apoptosis. A
specific inhibitor of an essential step in the
biochemical machinery that mediates apoptosis is needed.
25 Such an inhibitor would be a drug candidate for
therapeutic use against apoptosis-associated diseases.
Because RIP interaction with (i.e., binding to)
intracellular factors appears to be an essential step in
triggering apoptosis, inhibitors of RIP binding to
30 intracellular apoptosis factors, e.g., Fas/APO-1 or
TNFR1, are potential drug candidates. RIP (or a fragment
thereof) is an essential component in any screening
method for discovery of such RIP binding inhibitors.
       RIP inhibitor screening methods, including cell-
35 free methods and cellular methods, can be used in the

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practice of this invention. Cellular screening methods
within the scope of this invention can involve transient
expression vectors or stable transformation. Various RIP
inhibitor screening protocols can be designed, according
5   to well-known principles, by one of ordinary skill in the
art, once RIP and RIP-encoding DNA are in hand, by virtue
of the present invention.

Cell-free screening methods for inhibitors of RIP
death domain-mediated binding involve the use of isolated
10 RIP and an RIP interaction partner, e.g., isolated
Fas/APO-1 protein, TNFR1, or a polypeptide comprising the
appropriate binding domain of one of these proteins.
Soluble forms of RIP and RIP interaction partners can be
utilized in cell free RIP inhibitor screening protocols.
15 Preferably, however, the cell-free RIP inhibitor
screening protocol involves an RIP interaction partner
inserted into a biological membrane. Membrane-inserted
fusion protein forms of Fas/APO-1, can be produced, for
example, using known sequences, according to the methods
20 of Clement et al. (supra).

Preferably, RIP inhibitor screening is carried out
in a cellular system, using a reporter strain of cultured
mammalian cells, transformed with one or more vectors
encoding RIP, and other assay components, as necessary.
25 Preferably, an RIP-encoding sequence is cloned into a
recombinant DNA vector, where it is expressed under the
control of an inducible promoter, e.g., a heat shock
promoter. See, e.g., Wurm et al., Proc. Natl. Acad. Sci.
USA 83:5414, 1986. Following induction of RIP
30 expression, Cell death is measured in experimental
treatments involving the presence of an inhibitor
candidate, and in appropriate positive and negative
controls. Various assays for cell death are known in the
art, including the neutral red uptake method (Wallach, J.
35 Immunol. 132:2464, 1984), the crystal violet method (see,

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e.g., Itoh et al., *supra*), or microscopic inspection of cells for visual signs of apoptosis.

Because overexpression of RIP can be used to induce apoptotic cell death, RIP is useful as a tool in
5 gene therapy in at least two different ways: (1) to control the number of cells bearing a specific gene; and (2) to act as an anti-tumor agent in forms of cancer therapy that are dependent on the delivery of a lethal gene to neoplastic cells. In both applications,
10 overexpression of RIP to cause apoptotic cell death is preferable to approaches employing death-inducing genes that result in in vivo generation of toxic agents or that interfere with cell cycle progression.

Cell ablation through RIP expression is
15 advantageous because apoptotic death affects both mitotically active and mitotically quiescent cells. In contrast, chemotherapeutic agents and many gene therapy-based treatments for tumors require the target cell to be replicating in order for the treatment to be effective.
20 RIP genes used in gene therapy are preferably under the control of an exogenously regulatable promoter. An exogenously regulatable promoter is a promoter that can be induced by a specific set of environmental conditions, such as the increase in the concentration of
25 a specific inducer. Examples of exogenously regulatable promoters and inducing conditions include: induction of a metallothionein promoter by zinc ions (Makarove et al., Nucleic Acids Res. 22:1504-1505, 1994), removal of tetracycline, thereby activating a synthetic promoter
30 based on the action of a tetracycline repressor-VP16 chimera (Gossen et al., Proc. Natl. Acad. Sci. USA 89:5547-5551, 1992), addition of ecdysone (Christopherson et al., Proc. Natl. Acad. Sci. USA 89:6314-6318, 1992), or the synthetic progesterone antagonist mifepristone

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(Wang et al., Proc. Natl. Acad. Sci. USA 91:8180-8184, 1994).

The RIP-encoding DNA of this invention enables one of ordinary skill in the art to produce anti-RIP
5 antibodies. The RIP-encoding DNA is used to construct a vector encoding a fusion protein comprising an RIP moiety and an isolation-facilitating moiety, i.e., a moiety that can be readily isolated from contaminating proteins in an extract from a host cell used to express the fusion
10 protein. A preferred isolation-facilitating moiety is maltose binding protein. DNA encoding maltose binding protein is commercially available. A binding reagent specific for the isolation-facilitating moiety is used for convenient and efficient isolation of the RIP fusion
15 protein. For example, amylose chromatography is preferred for isolation of a fusion protein comprising maltose binding protein moiety. Following isolation, the RIP fusion protein is used to produce RIP-specific antibodies (polyclonal or monoclonal), according to
20 standard methods, described, for example, by Harlow et al.,. (*Antibodies - A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988).

The anti-RIP antibodies of the invention have several uses. For example, they may be used as reagents
25 for preparation of affinity chromatography media. Once the anti-RIP antibodies of this invention are in hand, preparation of RIP affinity chromatography media can be carried out according to conventional methods (*see, e.g.*, Harlow et al., *supra*), using commercially available
30 reagents. The RIP-specific affinity chromatography media can be used to isolate full-length RIP from natural sources or from host cells transformed with recombinant DNA encoding RIP.

The anti-RIP antibodies of the invention ar also
35 useful as analytical-scale laboratory reagents for

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research on the physiology and cell biology of apoptosis.
For example, immunohistochemical techniques, based on
anti-RIP monoclonal antibodies are likely to be valuable
tools for embryologists seeking ways to observe the rate
5   and/or distribution of apoptosis in the normal
morphological development of metazoan animals.

The anti-RIP antibodies of the invention are also
useful as diagnostic immunoassay reagents for measuring
RIP levels in tissue samples from patients suspected of
10 having an apoptosis-related disease or abnormality.
Information on RIP levels in selected cells or tissues is
a useful diagnostic or prognostic indicator in any
situation where the rate of programmed cell death is
important. The type of tissue sampled for the diagnostic
15 test will vary, depending on the signs and symptoms of
the patient and the suspected disease or abnormality.

If the tissue sample is highly homogenous with
respect to cell type, it may be preferable to carry out
the RIP immunoassay on an extract from a homogenate.
20 Alternatively, it may be preferable to use an
immunohistochemical assay involving anti-RIP antibodies.
An immunohistochemical assay is preferable when the
tissue sample is heterogenous with respect to cell type.
An immunohistochemical assay will yield information on
25 the distribution of differing RIP levels in a cross
section of tissue, or differing RIP levels in various
types of blood cells in a blood sample.

Although inhibitors of RIP binding to
intracellular apoptosis factors would be expected to have
30 therapeutic utility only for disease states involving
increased apoptosis, information on the level of RIP in a
tissue sample would have diagnostic/prognostic utility
for any apoptosis-related disease, regardless of whether
apoptosis was increased or decreased in that disease.
35 Examples of diseases associated with decreased apoptosis

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include cancer (in particular, follicular lymphomas,
carcinomas with p53 mutations, hormone-dependent tumors,
e.g., breast cancer, prostate cancer, ovarian cancer),
autoimmune disorders (e.g., systemic lupus erythematosus,
5 immune-mediated glomerulonephritis), viral infections,
herpes viruses, poxviruses, adenoviruses).

     The anti-RIP antibodies of the present invention
can be used in various diagnostic immunoassay formats
known in the art. Exemplary immunoassay formats are
10 competitive radioimmunoassay, ELISA, Western blot
analysis and microcapillary devices comprising
immobilized antibody. *See, e.g.*, Dafforn et al., Clin.
Chem. 36:1312, 1990; Li et al., Anal. Biochem. 166:276,
1987; Zuk et al., U.S. Patent No. 4,435,504; Zuk et al.,
15 Clin. Chem. 31:1144, 1985; Tom et al., U.S. Patent No.
4,366,241; and Clark, PCT published application WO
93/03176.

     The RIP-encoding DNA of this invention can be used
as an *in situ* hybridization reagent to assess
20 transcription of RIP genes and observe RIP RNA
processing, for diagnostic purposes or research purposes.

     The RIP-encoding DNA of this invention can be
obtained by screening a transcriptional activator fusion
library from the species of interest, by means of a
25 protein interaction system, as was done in the original
work on this invention, and which is described in detail,
below. Preferably, however, RIP-encoding DNA is obtained
through a more direct approach, using nucleotide
sequences or amino acid sequence information provided
30 herein.

     A general method for cloning a full length human
RIP cDNA involves screening a human cDNA library
(randomly primed from any tissue) with a probe derived
from the 5' region of the murine RIP coding sequence,

using a low stringency procedure similar to that described under the "cDNA Cloning" heading, below.

A wide variety of host/expression vector combinations can be employed for expressing RIP-encoding DNA of this invention. The expression of RIP-encoding DNA in a cellular screening assay is preferably in a eukaryotic cell, under the control of eukaryotic expression control sequences. More preferably, the eukaryotic cell is a cultured mammalian cell. If the expression of recombinant RIP-encoding DNA is merely for the production of isolated recombinant RIP, however, a prokaryotic host/expression vector system or a eukaryotic host/expression system can be used.

In the studies leading to the present invention, a yeast two-hybrid screen of a human T-cell cDNA library identified two proteins capable of interacting with the intracellular domain of Fas/APO-1: Fas/APO-1 itself and a novel protein, RIP, containing a death domain and a kinase domain. Neither protein interacts with a humanized variant of the murine lpr^{cg} allele, which confers much the same phenotype on affected mice as does the original lpr allele (Matsuzawa et al., 1990). The finding that the intracellular domain of the lpr^{cg} variant is incapable of interacting with the wild type Fas/APO-1 cytoplasmic domain in yeast suggests that oligomerization of Fas/APO-1 mediated by cytoplasmic sequences may be a prerequisite for activity. However it has not been established whether oligomerization of Fas/APO-1 is necessary for interaction with RIP, or whether the same subdomain exploited for oligomerization is also required for heteromeric association with RIP. The discovery of the Fas-Fas interaction in a library screen is consistent with a recent demonstration that TNFR1 and Fas/APO-1 are capable of both self- and cross-association in yeast (Boldin et al., J. Biol. Chem.

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270:387, 1995). The death domain probably mediates such
interactions, because work on this invention indicates
that RIP can also associate with the intracellular domain
of TNFR1 (as well as the intracellular domain of Fas/APO-
5 1).

RIP mRNA is expressed at low levels in all
tissues. A pattern of widespread expression has also
been observed for Fas/APO-1. In contrast to RIP,
however, Fas/APO-1 mRNA is found at higher levels in
10 thymus, liver, lung, and heart. Like Fas/APO-1 mRNA, RIP
mRNA is induced in splenocytes after activation with
ConA. Although a requirement for RIP in Fas-mediated
killing has not been established, coordinate induction of
Fas/APO-1 and RIP may contribute to increased
15 susceptibility of T-cells to Fas-mediated cell death
following activation.

The N-terminus of RIP contains a kinase domain
that has features of both Ser/Thr and Tyr kinases. In
the two interacting loops that appear to control
20 hydroxyamino acid recognition (Hubbard et al., Nature
372:746, 1994; Taylor et al., Structure 2:345, 1994), RIP
closely resembles a Ser/Thr kinase. In particular, it
lacks the Ala-X-Arg or Arg-X-Ala motif in the catalytic
loop (subdomain VI) and the Pro-X-X-Trip motif in the P+1
25 loop (subdomain VIII), both of which are found in all Tyr
kinases (see, e.g., Hanks et al., in Meth. Enzymol. 200,
Academic Press, at pp. 38-62, 1991).   Other support for
the possibility that RIP has either mixed catalytic
specificity or Tyr kinase-like regulation with Ser/Thr
30 specificity, comes from the finding that, among known
sequences, RIP shows the greatest overall primary
sequence similarity to murine lck, a tyrosine kinase of
the src family. The relatedness to tyrosine kinases is
especially apparent among the framework residues outside
35 the active site which make up the C-terminal, alpha

helic-rich substrate-binding lobe. For example, RIP has
a tryptophan at position 269 which is present in all Tyr
kinases analyzed by Hanks et al. (Science 241:42, 1988),
but absent from all Ser/Thr kinases examined, except Mos.

5   The presence of structural motifs from both Tyr and
Ser/Thr kinases has also been noted for the soybean
kinase GmPK6 (Feng et al., Biochim. Biophys Acta
1172:200, 1993), which shows high global similarity to
RIP. Although the exact role of protein kinases in

10 Fas/APO-1 mediated apoptosis is unknown, it appears that
the participation of at least one protein tyrosine kinase
is required.

       In work on this invention, overexpression of
RIP resulted in the induction of a cell death program

15 morphologically indistinguishable from apoptosis.
Deletion of the C-terminal region of RIP spanning the
segment of death domain homology eliminated the apoptotic
response, but deletion of the kinase domain did not
entirely quench activity.

20   Although the evidence that RIP binds to
Fas/APO-1 in yeast favors a model in which RIP acts
directly downstream of Fas/APO-1 in a death pathway, it
is also possible that RIP has other actions. For
example, the elevation of RIP mRNA in activated T-cells

25 suggests that RIP may be involved in the apoptosis
provoked by growth factor deprivation.

                Experimental Information

RIP cDNAs

             A cDNA library screen for proteins that

30 interact with the intracellular domain of Fas/APO-1 was
conducted by using a variation of the yeast interaction
system described by Gyuris et al. (Cell 75:791, 1993).
The assay system involved a yeast reporter strain
containing two separate reporter genes, *S. cerevisiae*

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leu-2 and *E. coli* β-galactosidase coding sequences under
the control of a synthetic promoter which contain a Lex-A
binding site, and which required a trans-acting
transcriptional activator in order for transcription to
5   occur. A cDNA segment comprising most of the cytoplasmic
domain of human Fas/APO-1 (residues 192 to 329 of the Fas
precursor) was fused to the 3' end of the coding region
for the bacterial repressor LexA, in a yeast expression
plasmid, which was used to transform the reporter strain.
10 This Fas/LexA construct encoded a transcriptionally inert
fusion protein ("the bait"), capable of specific binding
at the LexA binding site in the reporter gene promoters,
by virtue of the LexA moiety. The bait-expressing
reporter strain was transformed with a transcriptional
15 activator fusion protein library prepared from mRNA
isolated from the Jurkat (human T-cell leukemia) cell
line, which is known to undergo apoptosis when subjected
to treatment with anti-Fas antibody. The transcriptional
activator moiety of the library-encoded fusion proteins
20 lacked DNA binding activity. Any transcriptional
activator fusion protein with the ability to bind to the
Fas/APO-1 intracellular domain, however, would bring the
transcriptional activator moiety into proximity with the
reporter gene promoter, thereby resulting in
25 transcription of the reporter genes.

        Transformants were plated on selective (leucine
deficient) plates containing galactose, which induces the
GAL1 promoter that directs transcription of the library
insert. Leucine prototrophs were transferred to plates
30 containing X-gal and galactose, and colonies giving a
dark blue color reaction were recovered and analyzed
further.

        To test the specificity of the interaction
between the candidate interaction partners and Fas/APO-1,
35 the library plasmids were reintroduced into a second

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strain harboring a LexA-Fas fusion gene (LexA-lpr^cg) in which the Fas portion had been mutated by substitution of asparagine for valine at position 254 of the Fas/APO-1 precursor sequence. This mutation was expected to
5 exhibit a molecular phenotype similar to that of the murine lpr^cg allele, which is formed by substitution of asparagine for isoleucine at the homologous position. None of the candidate plasmids showing evidence of strong interaction with LexA-Fas were capable of interacting
10 with LexA-lpr^cg (Figure 1A; data not shown).

Restriction site and sequence analysis of the cDNA inserts of the candidate clones showed all to be incomplete cDNAs falling into only two classes. One of the inserts encoded the C-terminal residues 222 to 335 of
15 the intracellular domain of Fas/APO-1 itself. The other encoded a protein, designated RIP, which had no overt relationship to previously described polypeptides.

The specificity of the interaction partners was further tested by using the library plasmids to transform
20 yeast harboring expression plasmids encoding LexA fusions with intracellular domains of various cell surface receptors. Although no interaction was detected in most cases, weak interaction was detected following introduction of the yeast plasmid encoding Act-RIP into
25 strains harboring LexA-TNFR1 intracellular domain. By contrast, no activity was seen when Act-RIP was introduced into strains harboring LexA-TNFR2 intracellular domain. Yeast transformed with Act-Fas displayed promoter activity in strains harboring LexA-Fas
30 intracellular domain, but showed no activity in strains harboring any other LexA-intracellular domain.

To measure this effect more precisely, β-galactosidase assays were performed on lysates of yeast harboring various pairs of LexA-intracellular domain and
35 Act-RIP. Lysates from yeast bearing LexA-Fas and Act-RIP

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contained about 30 to 40 fold more β-galactosidase
activity than strains bearing Act-RIP and either LexA-
1pr^{cg} or LexA-TNFR2 (Fig. 1). Lysates prepared from yeast
harboring LexA-TNFR1 and Act-RIP expressed β-
5   galactosidase activity at about 10% of the level seen in
lysates prepared from yeast bearing LexA-Fas and Act-RIP
(Fig. 1).
          One explanation for the failure to detect an
interaction between Act-RIP and LexA-1pr^{cg} or LexA-TNFR2
10 could have been that the LexA fusion proteins were poorly
expressed. To address this possibility, a portion of
each lysate used to measure enzyme activity was subjected
to gel electrophoresis and blot transfer, followed by
detection with anti-LexA antiserum. LexA fusion proteins
15 of the appropriate size were detected in each of the
lysates and both the LexA-1pr^{cg} and LexA-TNFR2 fusion
proteins were found to be more abundantly expressed than
LexA-Fas or LexA-TNFR1 (data not shown), making it
unlikely that failure to detect interaction *in vivo* could
20 be attributed to degradation or inadequate synthesis of
the LexA chimeras.
<u>Cloning and Structure of Murine RIP</u>
          Because the RIP cDNA insert identified in the
two hybrid screen did not encode an open reading frame
25 bearing a consensus translational initiation sequence,
additional human cDNA libraries were screened by
hybridization for a full length clone. Initially, none
were identified, however, and several of the resulting
isolates appeared to terminate at approximately the same
30 5' terminus, suggesting that secondary structure in the
mRNA might have prevented extension of the cDNA by
reverse transcriptase. (Subsequently, overlapping cDNA
clones representing the entire human RIP coding sequence
were obtained.) Although the largest clone spanned
35 approximately 1 kb of sequence, preliminary RNA blot

hybridizations revealed a transcript of approximately 4.2 kb expressed in cell lines of diverse provenance, including tumors of lymphoid, hepatic, renal, neuronal, cervical, intestinal, muscular and skeletal origin.

5   To isolate a full length murine RIP clone, the human RIP coding sequence was used to probe a mouse thymus cDNA library. Four distinct overlapping clones were identified, ranging in size from 1 kb to 2.4 kb, as shown in Fig. 2. Restriction analysis and sequencing

10 revealed that one of these clones, III2, extended further 5′ than the others. The 2.3 kb insert of III2 contains a 1968 nucleotide open reading frame beginning with a translational initiation consensus sequence and predicting a polypeptide of 656 amino acids (SEQ ID

15 NO:15) with M_r of 74,000 (Fig. 3). The nucleotide sequence of a full-length murine RIP cDNA is (SEQ ID NO:14).

        The amino terminal region of RIP bears an extended homology to protein kinases that begins a few

20 residues after the presumptive initiating methionine and extends to the vicinity of residue 300. Quantitative sequence comparisons based on a word match algorithm (Altshul et al., J. Mol. Biol. 215:403, 1990) predict that this domain is most similar overall to the tyrosine

25 subclass of protein kinases, with the highest relatedness seen to the mouse *lck* gene product (Marth et al., Cell 43:393, 1985). However in the key subdomains that discriminate most closely between tyrosine and serine/threonine substrate specificity, the HKDLKPEN

30 motif of the catalytic loop (kinase subdomain VI) and the GTLYYMAPE sequence of the P+1 loop (kinase subdomain VIII), RIP appears to match the serine/threonine family consensus (Hanks et al., in Methods in Enzymology vol. 200, (Hunter et al., eds.), Academic Press, pp. 38-62,

35 1988; Taylor et al.; supra).

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The sequence predicted by one of the human cDNA
fragments consists of 375 amino acids corresponding to
the region just C-terminal to the kinase domain of murine
RIP, and shares 67% sequence identity with the murine
5 sequence over this length. Within this portion, the
first 270 amino acids following the kinase domain have no
striking homology to other proteins, although a small
subdomain within this portion is highly conserved between
mouse and human proteins (residues 391 to 427 of the
10 murine sequence) and has a relatively high representation
of Arg (R), Gln (Q) and Glu (E) (18/37 residues in both
sequences). The sequence of this region is similar to
portions of the trichohyalin family of hair structural
proteins, which contain RQE-rich repeats that form highly
15 stabilized alpha helices (Lee et al., J. Biol. Chem.
268:12164, 1993).

Complete Human RIP Coding Sequence

Subsequent to the cloning and sequencing of a
full-length murine RIP cDNA (SEQ ID NO:14), a complete
20 human RIP coding sequence was obtained from sequence
analysis of multiple overlapping partial cDNAs. A
complete human RIP coding sequence is (SEQ ID NO:16).
The deduced amino acid sequence of a full-length human
RIP is (SEQ ID NO:17).

25 RIP C-terminus Death Domain Homology

The remaining 98 C-terminal amino acids share
87% sequence identity between mouse and human RIP,
suggesting they subserve some regulatory function (Fig.
3). Comparable domains of approximately 90 residues
30 close to the C-termini of Fas/APO-1 and TNFR1 have been
shown to play a role in the transduction of apoptotic
signals to receptive cells, and have been termed "death
domains" for this reason (Tartaglia et al., Cell 74:845,
1993). Pairwise comparisons of the death domain

sequences aligned in Fig. 4 showed the highest
relatedness between human RIP and human TNFR1 (59%
similarity and 30% identity), which are significantly
more similar than human Fas/APO-1 and human TNFR1 (42%
5   similarity and 23% identity). The interspecies
conservation of the RIP death domain (85% identity
between mouse and human) exceeds that of the TNFR1 (68%
identity) and Fas/APO-1 (49% identity) death domains.
Constitutive and Inducible Expression of RIP mRNA

10   Preliminary RNA blot hybridization experiments
demonstrated the existence of an RNA species of
approximately 3.8 kb in a variety of cell lines. To more
precisely assess mRNA abundance in tissues a quantitative
ribonuclease protection assay was employed. Use of a
15   labeled antisense RNA probe corresponding to the 3'
terminus of the cDNA gave rise to a ribonuclease
resistant species of the expected size in all adult
tissues tested. An *in vitro* labeled RNA antisense to the
mRNA for the ribosomal large subunit protein L32 was used
20   as an internal standard to allow normalization to the
amount of RNA loaded in each lane. Analysis of the
protected RNA showed that RIP mRNA levels varied by less
than 2-3 fold between most tissues (not shown); lung
showed the highest expression, whereas tongue showed the
25   least.

          The possibility that RIP mRNA might be
regulated as a consequence of activation in T-cells was
also explored. Dissociated murine splenocytes were
stimulated *in vitro* with the lectin concanavalin A and
30   total RNA prepared at various times following addition of
lectin was analyzed for the presence of RIP sequences by
RNA blot analysis. Little or no RIP RNA could be
detected in unstimulated splenocytes, but a single 3.6-
3.8 kb species appeared in unfractionated splenocytes
35   that had been exposed to lectin for 2 h or longer. Since

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RIP mRNA is detectable by ribonuclease protection in the
spleen as a whole, the inability to detect RIP mRNA in
splenocytes treated with ConA for less than 2 h is
probably due to the lower sensitivity of RNA blot
5 analysis, although this discrepancy could also have
resulted from RIP expression exclusively in the fibrous
tissue of the spleen.

Immunodetection of RIP Produced In Vivo

       To examine the distribution of RIP protein *in*
10 *vivo*, a rabbit antiserum was prepared against a fusion
protein consisting of the 250 C-terminal residues of
murine RIP fused to *E. coli* maltose binding protein. The
antiserum specificity was validated by
immunoprecipitation of RIP synthesized *in vitro*.
15 Following *in vitro* transcription and translation of the
RIP open reading frame, a single labeled product of
approximately 74 kD was observed which could be
specifically immunoprecipitated with the rabbit
antiserum, but not with serum from unimmunized animals.
20 The specificity of the antiserum for RIP was also
documented by its inability to immunoprecipitate an
irrelevant protein (*P. pyralis* luciferase) similarly
translated *in vitro*. Immunoprecipitation of a lysate of
metabolically labeled NIH3T3 cells with the rabbit
25 antiserum revealed the presence of a single protein
species with the same molecular mass as that revealed by
in vitro translation of RIP.

Detection of RIP in Transiently Transfected BHK Cells

       To determine whether RIP protein could have a
30 direct effect on cell viability, BHK cells grown on
coverslips were transiently transfected with a epitope-
tagged version of RIP (RIPmyc). Transfected cells were
reacted with anti-RIP antiserum or an anti-myc monoclonal
antibody. Weak expression was detected with both
35 antibodies. The pattern of immunoreactivity was

heterogeneous, with both diffuse cytoplasmic as well as punctate perinuclear patterns observed. DNA staining with Hoechst 33258 showed that many of the RIP-expressing cells had apoptotic nuclei, a feature not seen when

5   vector or Fas control expression plasmids were used. However, a number of RIP-expressing cells could be found that had normal-appearing nuclei; conversely, apoptotic cells having no detectable RIP staining were also seen.
     The concordance of cell death and RIP

10 expression suggested the ability to detect RIPmyc protein might be compromised by the death of the cells in which it was being expressed. To test this, and the possible role of individual RIP domains in apoptosis, two additional epitope-tagged constructs were prepared: one

15 lacking C-terminal sequences, including the death domain (RIPmycΔdeath), and one lacking ~ 200 amino acids in the kinase domain (RIPmycΔkinase). Both deletion mutants showed greater immunoreactivity with anti-myc antibodies than the full length construct. Only RIPmycΔkinase was

20 detected by the anti-RIP antiserum, as expected from the deletion of its epitope from RIPmycΔdeath.

RIP Overexpression Leads to Cell Death

     To determine whether RIP was inducing cell death, we marked the transfected cells by co-transfection

25 with β-galactosidase. Cells were transfected with pairs of expression plasmids encoding RIPmyc and β-galactosidase (Price et al., 1987) at a 1:3 ratio of β-gal plasmid to RIP plasmid. After histochemical detection of β-galactosidase activity, cells transfected

30 with β-gal and RIPmyc expression plasmids were found to contain a large proportion of intensely staining, shrunken blue cells that exhibited membrane blebbing and loss of adherence. By contrast, transfection with β-gal plasmid, either alone or in combination with RIPmycΔdeath

35 plasmid, had no adverse effect upon nuclear morphology

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and resulted in a predominantly cytoplasmic β-
galactosidase staining pattern. Co-transfection of β-gal
plasmid with RIPmycΔkinase likewise gave cytoplasmic
staining of healthy-appearing cells, although a number of
5 shrunken, blebbed cells were also seen.

        To quantify these results, cells from three
independent transfections were examined and the
morphologically apoptotic blue cells enumerated as a
fraction of total blue cells. Over 57 percent of blue
10 cells arising from co-transfection of RIPmyc and the β-
gal plasmid showed morphological changes consistent with
apoptosis, whereas only 1-2 percent of blue cells that
had been transfected with β-gal plasmid alone or in
combination with RIPmycΔdeath exhibited such a phenotype.
15 However a consistent low frequency of apoptotic changes
was seen in co-transfections involving RIPmycΔkinase,
with almost 11 percent of the blue cells appearing to
have undergone cell death. The fraction of cells showing
morphological changes was positively correlated with the
20 ratio of RIPmyc or RIPmycΔkinase plasmid to β-gal
plasmid, so that increasing RIP:β-gal plasmid ratios gave
higher percentages of dead blue cells (data not shown).
Plasmid Construction

        The yeast interaction system was modified from
25 that described by Gyuris et al. (supra) by engineering
the LexA expression plasmid to remove an internal MluI
site and to insert MluI and NotI sites downstream from
the DNA portion encoding the C-terminus of the gene. The
resulting distal polylinker has the site sequence MluI-
30 PmeI-NotI-EcoRI in the frame in which the MluI site
encodes Thr and Arg (frame 1).

        The DNA encoding receptor cytoplasmic tails
were amplified by PCR from cDNA libraries and cloned as
MluI-NotI or BssHII-NotI fragments using the following
35 oligonucleotide primers: Fas: 5'-CGCGGGACGC GTAAGGAAGT

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ACAGAAAACA TGC-3' (SEQ ID NO:1) and 5'-CGCGGGGCGG
CCGCTCTAGA CCAAGCTTTG GATTTC-3' (SEQ ID NO:2); TNFR1: 5'
CGCGGGGCGC GCTACCAACG GTGGAAGTCC AAG-3' (SEQ ID NO:3) and
5' CGCGGGGCGG CCGCTGCCCG CAGGGGCGCA GCCTCA-3' (SEQ ID
5 NO:4); and, TNFR2: 5'-CGCGGGACGC GTAAGAAGCC CTTGTGCCTG
CAG-3' (SEQ ID NO:5) and 5'-CGCGGGGCGG CCGCTTTAAC
TGGGCTTCAT CCC AGC-3' (SEQ ID NO:6).

　　　　The Fas/APO-1 cytoplasmic domain used in the
library screen diverges at the Glu located five residues
10 prior to the C-terminus, and continues an additional 25
residues through vector sequences to the C-terminus. In
all subsequent analyses, these residues were found not to
contribute detectably to either Fas-Fas or Fas-RIP
interaction.

15 　　　　A mutant Fas bait protein analogous to the lpr^{cg}
point mutation was made by mutating the valine at
position 254 of human Fas to asparagine, using the
following oligonucleotides in a recombinant PCR reaction:
　　　5'-CGAAAGAATG GTAACAATGAA GCC-3' (SEQ ID NO: 7), and
20 　　　5'-GGCTTCATTG TTACCATTCTT TCG-3' (SEQ ID NO: 8).
In the resulting construct, residues 330 and 331 were
also converted from Glu and Ile to Gly and Asn,
respectively.

　　　　A myc-tagged version of RIP (RIPmyc) was made
25 by digesting RIP clone III2 with TfiI and ligating a
HindIII/TfiI adaptor to the 5' end and TfiI/NotI adaptor
containing the myc epitope and a stop codon to the 3'
end. The sense (#145) and antisense (#146)
oligonucleotides comprising the 3' adaptor were as
30 follows:
　　　#145: 5'-ATTCGTGCCA GCCAGAGCGG CATGGAGCAG AAGCTCATCT
　　　　　　　CAGAAGAAGA CCTCGCGTAA GC-3' (SEQ ID NO:9), and

　　　#146: 5'-GGCCGCTTAC GCGAGGTCTT CTTCTGAGAT GAGCTTCTGC
　　　　　　　TCCATGCCGC TCTGGCTGGC ACG-3' (SEQ ID NO:10).

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The resulting insert was cloned into the HindIII and NotI sites of pcDNA I (Invitrogen).

To make RIPmycΔkinase, a PCR reaction was performed using a 5' RIP primer (#160: 5'-CCCAAGCTTG TTGGAGATTC TGAGCAATC-3' (SEQ ID NO:11)) and an internal kinase domain primer (#161: 5'-CCCGATCTGC AGGTCATGTA AGTCGCACATGCC-3 (SEQ ID NO:12)). The resulting product was cloned into the HindIII and PstI sites of RIPmyc resulting in the deletion of RIP residues 132 to 323.

RIPmycΔdeath was made by PCR, using a T7 primer and a myc-tag-containing primer (#151: 5'-CCCCTCGAGT TAGAGGTCTT CTTCTGAGAT GAGCTTTTGC TCTTTCTTTA AACTTGCCAC-3' (SEQ ID NO: 13)). The amplified RIPmycΔdeath sequence, lacking amino acids 309 to 656, was subcloned as a HindIII/XhoI fragment into a pcDNA I. Myc tags were located at the C-terminus of all proteins, and thus detection of the myc epitope requires translation of the entire cloned sequence. The BAG retrovirus vector encoding β-galacto-sidase has been described (Price et al., Proc. Natl. Acad. Sci. USA 84:156, 1987).

<u>Yeast Strains and Library Screen</u>

Yeast transformation with library DNA was performed according to the method of Schiestl and Gietz (Curr. Genet. 16:339, 1989). Recipient cells, EGY48/pRB1840 (Gyuris et al, supra) bearing LexA-fusion protein plasmids, were grown overnight in YPAD medium to a density of approximately 10^7 cells/ml, then diluted in 100 ml of warmed YPAD to a density of $2x10^6$ cells/ml and regrown to 10^7 cells/ml. The cells were harvested and washed in water, resuspended in 1 ml water, transferred to a sterile microcentrifuge tube and pelleted. The pellet was resuspended in 0.5 ml of 10mM Tris HCl (pH 7.5), 1 mM EDTA, 0.1 M Li acetate, (pH adjusted to 7.5 with acetic acid and passed through 0.2 micron filter). 50 μl of the resulting suspension was mixed with 1 μg of

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transforming DNA and 50 μg of single stranded salmon
sperm DNA, after which 0.3 ml of a solution of 40%
polyethylene glycol-4000 in Tris/EDTA/LiOAc was added and
mixed thoroughly, followed by incubation at 30°C with
5   agitation for 30 min. After a heat pulse at 42°C for 15
min, the cells were pelleted in a microcentrifuge and the
pellets resuspended in 1 ml of Tris/EDTA, diluted and
plated on selective medium. Library screening and
recovery of plasmids was performed as described by Gyuris
10 et al. (supra).
         For assessing the interaction of RIP and Fas
with other bait proteins, cells of the yeast strain
EGY48/pSH18-34, containing eight copies of the LexA
operator-lacZ reporter, were transformed with each of the
15 four bait constructs and selected on Ura⁻His⁻ glucose
plates. These bait strains were subsequently used for
transformation of the RIP or Fas library plasmids and
plated on Ura⁻His⁻Trip⁻Xgal plates containing either 2%
glucose of 2% galactose/1% raffinose. The LexA-TNFR2
20 bait strain gave a weak blue color reaction when grown on
galactose, indicating spontaneous transcriptional
activation by the fusion protein.
β-Galactosidase Assays and Immunoblotting
         Assays of crude extracts were carried out as
25 described (Rose et al., 1990). In brief, cells bearing
the appropriate bait and interaction plasmid were grown
to saturation overnight at 30°C in minimal Ura⁻His⁻Trip⁻
medium with 2% glucose. The next day, cells were diluted
1:50 into medium containing 2% galactose and 2% raffinose
30 and allowed to grow overnight. Cells were spun and
resuspended in breaking buffer (100 mM Tris (pH 8), 20%
glycerol (v/v), 1 mM DTT). Half of each suspension was
transferred to a separate tube, and PMSF was added to a
final concentration of 10 mM. Cells were lysed by
35 vortexing with acid-washed beads, and the resulting

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lysate was cleared by centrifugation. Enzyme activity
was measured by incubating in Z buffer (Miller,
Experiments in Molecular Genetics, Cold Spring Harbor
Laboratory, 1972) with 0.67 mg/ml o-nitrophenyl-β-D-
5 galactoside substrate. Reactions were stopped with 0.5
ml 1M Na_2CO_3 when an appropriate level of color had
developed. The protein concentrations of the lysates
were determined by Bradford assay. Units of β-
galactosidase were calculated using the following
10 equation: specific activity
(nmoles/min/mg)=$(OD_{420}.378)$/(time(min).vol
extract(ml).concentration (mg/ml)).

cDNA Cloning

Additional clones overlapping the primary RIP
15 isolate were sought in two libraries -- an expression
library prepared in the CDM8 plasmid vector using mRNA
isolated from the human cytolytic T-cell line WH3, and
from a commercially available human leukocyte library in
lambda phage (Clontech; HL1169a). Both libraries were
20 screened by filter replica hybridization, using
radiolabeled probes derived from the insert isolated by
interaction screening, as well as from subsequent inserts
identified by hybridization.

To isolate murine RIP cDNA clones, 1.2 kb of
25 human RIP sequence was subcloned into two halves and each
fragment used to probe genomic DNA blots containing mouse
and human DNA to optimize conditions for cross-species
hydrization. A commercially available oligo(dT)-primed
mouse thymus cDNA library (Stratagene) from a (C57B1/6 X
30 CBA)F_1 mouse was plated out, and 10^6 plaques were screened
using each of the human fragments on duplicate Genescreen
filters (DuPont). Probes were synthesized using the
random hexamer method Feinberg et al., Anal. Biochem.
132:6, 1983). Hybridization conditions were 5X SSPE, 10X
35 Denhardts, 2% SDS, 0.1 mg/ml herring sperm DNA at 55°C

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overnight. Filters were washed in 2X SSC, 0.1% SDS at
55°c with several changes over 1 h. Plaque purified
phage were isolated with three rounds of screening
(Sambrook et al.; supra), and *in vivo* excision carried
out using Exassist phage and SOLR™ recipient cells
(Stratagene). Seven independent clones were isolated,
and they fell into the four classes shown in Fig. 2. The
coding sequence of RIP was obtained as a composite from
the cDNA clones sequenced on both strands using Sequenase
T7 polymerase (U.S. Biochemical). Several nucleotide
polymorphisms were detected between the multiple clones,
only one of which resulted in an amino acid difference:
a Thr to Ile at position 473. Sequence comparisons were
done with Genetics Computer Group, Inc. or MegAlign
(DNAStar, Inc.) software using default parameters.

RNAse Protections and RNA Blot Hybridization

Tissue RNA samples were prepared from wild type
FVB mice (Taconic) by guanidinium thiocyanate lysis and
centrifugation through a CsCl cushion (Chirgwin et al.,
Biochemistry 18:5294, 1979). An antisense probe for RIP
made from cDNA clone III1, linearized with SpeI, was
synthesized using T7 polymerase with an *in vitro*
transcription kit (Stratagene), with the addition of 20
μM cold rUTP and 100 μCi ^{32}P-UTP (New England Nuclear).
The ribosomal L32 probe was synthesized from an XbaI-
linearized template at one tenth the specific activity of
the RIP probe. The use of L32 as an internal control for
RNA loading has been described elsewhere (Shen and Leder,
Proc. Natl. Acad. Sci. USA, 89:8240, 1992). As expected,
the *in vitro* transcription products were slightly larger
than the protected fragments of 525 and 279 nucleotides,
respectively. After removal of the DNA template with
DNAseI, probes were purified using an ultrafree-MC 30,000
retention filter (Millipore). A sample (2x10^5 cpm) of
the RIP probe and a sample of L32 (2x10^4 cpm) were mixed

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with 10 μg of RNA from each tissue or 50 μg of tRNA and
allowed to hybridize in PIPES buffer with 50% formamide
overnight at 50°C. Following this incubation,
unhybridized RNA was digested with RNAse A (40 μg/ml) and
5 RNAse T_1 (1.3 μg/ml) at 37°C for 15 min. Samples were
extracted with phenol/chloroform once, ethanol
precipitated, washed, and boiled for 3 min before loading
on a 6% polyacrylamide gel. After electrophoresis, gels
were dried and exposed and radioactivity was quantitated
10 with the aid of a Molecular Dynamics Phosphorimager.

    For measuring RIP in activated T-cells, spleens
were removed from adult animals and dissociated using a
stainless steel mesh. Cells ($\sim 10^7$ per timepoint) were
exposed to ConA (10 μg/ml) for 0-4.5 h, and RNA prepared
15 as described above. Approximately 8 μg of total RNA from
each sample was loaded on a 0.9% agarose/4% formaldehyde
gel, electrophoresed in 20 mM MOPS, 5 mM sodium acetate,
1 mM EDTA, and transferred to nylon filters (GeneScreen,
DuPont) by capillary transfer (Sambrook et al., supra).
20 Sizes were estimated using a molecular weight standard
(Gibco BRL). The blot was hybridized with either an RIP
probe corresponding to the C-terminal half of the protein
(cDNA III1) or with a probe detecting ribosomal 28S RNA.
Hybridization was allowed to proceed overnight in 40%
25 formamide, 4X SSC, 10% dextran sulfate, 7 mM Tris (pH
7.6), and 20 μg/ml salmon sperm DNA at 42°C. The blots
were then washed at 50°C in 1X SSC, 0.1% SDS (RIP probe)
or 60°C in 0.1X SSC, 0.1% SDS (28S probe), and exposed to
film with intensifying screens.
30 RIP Antisera and Immunoprecipitations
    Rabbit polyclonal antisera recognizing RIP were
prepared by Pocono Rabbit Farm & Laboratory, Inc.
(Canadensis, PA) using a fusion protein containing the C-
terminal 250 amino acids of mouse RIP fused to maltose-
35 binding protein (MBP; New England Biolabs). MBP-RIP was

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purified by amylose chromatography and acrylamide gel
elution. For immunoprecipitations, RIP was first
transcribed in vitro using 15 μg full-length RIP template
in a 300 μl reaction containing 3.3 mM each ATP, GTP,
CTP, and UTP, 280 units RNAse inhibitor, 400 units Sp6
RNA polymerase and 1X Sp6 buffer (Boehringer Mannheim
Biochemicals) at 37°C for 2 h. Translations were carried
out using a reticulocyte lysate kit containing a
luciferase positive control (Oromega) using approximately
4 μCi ^{35}S-Met per reaction. Reaction products were
diluted to 1 ml in a modified RIPA buffer (0.5% NP40,
0.5% sodium deoxycholate, 0.025% SDS, 50 mM Tris, 30 mM
sodium pyrophosphate, 50 mM NaF, 100 uM sodium
orthovanadate [pH7.6]) containing protease inhibitors and
precleared with 50 μl normal rabbit serum and 100 μl
protein A sepharose (1:1). Reactions were split in half, ·
5 μl preimmune or immune serum was added to each, and
samples were allowed to rotate for 1 h at 4°C. Complexes
were precipitated with 40 μl protein A sepharose (1:1),
washed 3 times with modified RIPA buffer, and resolved by
SDS-PAGE.

For immunoprecipitation from metabolically
labeled cells, a 150 mm plate of subconfluent NIH3T3
cells were incubated overnight in methionine-deficient
DMEM (Gibco/BRL, catalog #11970-019) supplemented with
^{35}S-methionine (~100 μCi/ml), 5% dialyzed fetal calf
serum, 2 mM glutamine, penicillin (50 U/ml), and
streptomycin (50 μg/ml). Cells were lysed for 15 min on
ice in lysis buffer (250 mM NaCl, 50 mM HEPES-KOH, [pH
7.5], 5 mM EDTA, 0.1 mM sodium orthovanadate, 50 mM NaF,
0.1% Triton-X, 100 μg/ml PMSF, 2 μg/ml pepstatin, and 10
μg/ml each aprotonin and leupeptin), scraped, and spun at
15,000 x g for 10 min. Cleared lysate was precleared
once with normal rabbit serum and twice with protein A-
sepharose (Pharmacia) before addition of antibodies (3 μl

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of preimmune or immune rabbit serum). Immune complexes
were harvested with protein A-sepharose, and beads were
washed four times with lysis buffer before resuspension
in denaturing sample buffer. Following SDS-PAGE, the gel
5   was enhanced for fluorography with Enlightening solution
(DuPont), and dried down on absorbent paper.

Transfections and Immunofluorescence

     BHK cells were plated the night before
transfection at a density of 10^4 cells per 18mm round
10   coverslip (VWR Scientific). $CaPO_4$ precipitates were made
by standard techniques and 18-20 h after transfection,
cells were analyzed by immunofluorescence, as described
by Heald et al. (Cell 74:463, 1993). Cells were fixed
for 10 minutes at 3% formaldehyde and washed extensively
15   in PBS containing 0.1% NP40. Cells were reacted with
primary and secondary antibodies for 30 minutes each.
Nuclei were stained with Hoechst 33258 (1 μg/ml) and
mounted on slides with 90% glycerol, 0.2 M Tris (pH 8.0).
The 9E10 anti-myc antibody (Evan et al., 1985) was
20   obtained as an ascites from the Harvard Cell Culture
facility and used at 20 μg/ml. RIP and β-galactosidase
antisera were used at dilutions of 1:200. FITC anti-
mouse and Texas Red anti-rabbit secondary antibodies were
obtained from Jackson Immunoresearch. Cells were
25   examined with a Zeiss Axiophot* fluorescent microscope.

     β-Galactosidase activity in cells was
visualized by fixing cells with 0.5% glutareldehyde for
15 min. followed by extensive washing in PBS containing 5
mM $MgCl_2$. Cells were stained in PBS containing 20 mM
30   each $K_3Fe(CN)_6$ and $K_4Fe(CN)_6 \cdot 3H_2O$; 1 mM $MgCl_2$, and 1 mg/ml
X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) until
a suitable color developed, usually for 2-3 hours. To
enumerate the fraction of blue cells which had undergone
apoptotic changes, cells were transfected with one of the
35   four plasmid combinations described and fixed 16 h after

transfection.   Blue cells were included for analysis only
if their morphological status could be scored
unambiguously.

Other embodiments of the invention are within
5 the following claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT: President and Fellows of Harvard College

  (ii) TITLE OF INVENTION: CELL DEATH PROTEIN

 (iii) NUMBER OF SEQUENCES: 35

  (iv) CORRESPONDENCE ADDRESS:
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   (v) COMPUTER READABLE FORM:
     (A) MEDIUM TYPE: Floppy disk
     (B) COMPUTER: IBM PC compatible
     (C) OPERATING SYSTEM: PC-DOS/MS-DOS
     (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

  (vi) CURRENT APPLICATION DATA:
     (A) APPLICATION NUMBER: PCT/US96/----
     (B) FILING DATE: 18-APR-96
     (C) CLASSIFICATION:

  (vi) PRIOR APPLICATION DATA:
     (A) APPLICATION NUMBER: 08/444,005
     (B) FILING DATE: 18-MAY-95
     (C) CLASSIFICATION:

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     (C) REFERENCE/DOCKET NUMBER: 00383/026001

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     (B) TELEFAX: 617/542-8906
     (C) TELEX: 200154

(2) INFORMATION FOR SEQ ID NO:1:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 33 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGCGGGACGC GTAAGGAAGT ACAGAAAACA TGC   33

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(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGCGGGGCGG CCGCTCTAGA CCAAGCTTTG GATTTC   36

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGCGGGGCGC GCTACCAACG GTGGAAGTCC AAG   33

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGCGGGGCGG CCGCTGCCCG CAGGGGCGCA GCCTCA   36

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCGGGACGC GTAAGAAGCC CTTGTGCCTG CAG   33

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 36 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCGGGGCGG CCGCTTTAAC TGGGCTTCAT CCCAGC   36

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 24 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGAAAGAATG GTAACAATGA AGCC   24

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 24 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGCTTCATTG TTACCATTCT TTCG   24

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 62 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATTCGTGCCA GCCAGAGCGG CATGGAGCAG AAGCTCATCT CAGAAGAAGA CCTCGCGTAA   60

GC   62

(2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 63 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGCCGCTTAC GCGAGGTCTT CTTCTGAGAT GAGCTTCTGC TCCATGCCGC TCTGGCTGGC   60

ACG   63

(2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 29 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

                                                                      29
CCCAAGCTTG TTGGAGATTC TGAGCAATC

(2) INFORMATION FOR SEQ ID NO:12:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 33 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

                                                                      33
CCCGATCTGC AGGTCATGTA AGTAGCACAT GCC

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(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 60 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCCCTCGAGT TAGAGGTCTT CTTCTGAGAT GAGCTTTTGC TCTTTCTTTA AACTTGCCAC   60

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 2268 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CATACTGAGC AAGAACCAAA AGTGGTGTGT TGGAGATTCT GAGCAATCAA AATGCAACCA   60

GACATGTCCT TGGACAATAT TAAGATGGCA TCCAGTGACC TGCTGGAGAA GACAGACCTA   120

GACAGCGGAG GCTTCGGGAA GGTGTCCTTG TGTTACCACA GAAGCCATGG ATTTGTCATC   180

CTGAAAAAAG TATACACAGG GCCCAACCGC GCTGAGTACA ATGAGGTTCT CTTGGAAGAG   240

GGGAAGATGA TGCACAGACT GAGACACAGT CGAGTGGTGA AGCTACTGGG CATCATCATA   300

GAAGAAGGGA ACTATTCGCT GGTGATGGAG TACATGGAGA GGGCAACCT GATGCACGTG   360

CTAAAGACCC AGATAGATGT CCCACTTTCA TTGAAAGGAA GGATAATCGT GGAGGCCATA   420

GAAGGCATGT GCTACTTACA TGACAAAGGT GTGATACACA AGGACCTGAA GCCTGAGAAT   480

ATCCTCGTTG ATCGTGACTT TCACATTAAG ATAGCCGATC TTGGTGTGGC TTCCTTTAAG   540

ACATGGAGCA AACTGACTAA GGAGAAAGAC AACAAGCAGA AGAAGTGAG CAGCACCACT   600

AAGAAGAACA ATGGTGGTAC CCTTTACTAC ATGGCACCCG AACACCTGAA TGACATCAAT   660

GCAAAGCCCA CGGAGAAGTC GGACGTGTAC AGCTTTGGCA TTGTCCTTTG GGCAATATTT   720

GCAAAAAAGG AGCCCTATGA GAATGTCATC TGTACTGAGC AGTTCGTGAT CTGCATAAAA   780

TCTGGGAACA GGCCAAATGT AGAGGAAATC CTTGAGTACT GTCCAAGGGA GATCATCAGC   840

CTCATGGAGC GGTGCTGGCA GGCGATCCCA GAAGACAGGC CAACATTTCT TGGCATTGAA   900

GAAGAATTTA GGCCTTTTTA CTTAAGTCAT TTTGAAGAAT ATGTAGAAGA GGATGTGGCA   960

```
AGTTTAAAGA AAGAGTATCC AGATCAAAGC CCAGTGCTGC AGAGAATGTT TTCACTGCAG    1020

CATGACTGTG TACCCTTACC TCCGAGCAGG TCAAATTCAG AACAACCTGG ATCGCTGCAC    1080

AGTTCCCAGG GGCTCCAGAT GGGTCCTGTG GAGGAGTCCT GGTTTTCTTC CTCCCCAGAG    1140

TACCCACAGG ACGAGAATGA TCGCAGTGTG CAGGCTAAGC TGCAAGAGGA AGCCAGCTAT    1200

CATGCTTTTG GAATATTTGC AGAGAAACAG ACAAAACCGC AGCCAAGGCA GAATGAGGCT    1260

TACAACAGAG AGGAGGAAAG GAAACGAAGG GTCTCTCATG ACCCCTTTGC ACAGCAGAGA    1320

GCTCGTGAGA ATATTAAGAG TGCAGGAGCA AGAGGTCATT CTGATCCCAG CACAACGAGT    1380

CGTGGAATTG CAGTGCAACA GCTGTCATGG CCAGCCACCC AAACAGTTTG AACAATGGA     1440

TTGTATAATC AGCATGGATT TGGAACTACA GGTACAGGAG TTTGGTATCC GCCAAATCTA    1500

AGCCAAATGT ATAGTACTTA TAAAACTCCA GTGCCAGAGA CCAACATACC GGGAAGCACA    1560

CCCACCATGC CATACTTCTC TGGGCCAGTA GCAGATGACC TCATAAAATA TACTATATTC    1620

AATAGTTCTG GTATTCAGAT TGGAAACCAC AATTATATGG ATGTTGGACT GAATTCACAA    1680

CCACCAAACA ATACTTGCAA AGAAGAGTCG ACTTCCAGAC ACCAAGCCAT CTTTGATAAC    1740

ACCACTAGTC TGACTGATGA ACACCTGAAC CCTATCAGGG AAAACCTGGG AAGGCAGTGG    1800

AAAAACTGTG CCCGCAAGCT GGGCTTCACT GAGTCTCAGA TCGATGAAAT CGACCATGAC    1860

TATGAAAGAG ATGGACTGAA AGAGAAAGTT TACCAAATGC TTCAGAAGTG GCTGATGCGG    1920

GAAGGCACCA AAGGGGCCAC AGTGGGAAAG TTGGCCCAGG CACTTCACCA ATGTTGCAGG    1980

ATAGACCTGC TGAACCACTT GATTCGTGCC AGCCAGAGCT AAGCCTGGGC AGGCTCTGGC    2040

AGTGGGAAGC AAACTATTTG TCTGGTGCAC AAACCCCGTT TGCCCACTAG CCTTCAGAAC    2100

TCTATCTCAG CATGAGCTCT GCATTTGAGC ACACAGGGTC ATGCAGTTTG GAACTGGTGG    2160

ATGGGAAGAG AAATCTGAAG CCCACAGTGA TTCTTCAGAA CATCCAAGCA TAAAGACCGC    2220

TGAATGAATG GTCGGTCCAT GACCAGTAGG AGAAAAAAAA AAAAAAAG                2268
```

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 656 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Gln Pro Asp Met Ser Leu Asp Asn Ile Lys Met Ala Ser Ser Asp
1               5                   10                  15
```

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```
Leu Leu Glu Lys Thr Asp Leu Asp Ser Gly Gly Phe Gly Lys Val Ser
         20              25                    30

Leu Cys Tyr His Arg Ser His Gly Phe Val Ile Leu Lys Lys Val Tyr
         35              40                    45

Thr Gly Pro Asn Arg Ala Glu Tyr Asn Glu Val Leu Leu Glu Glu Gly
    50              55                  60

Lys Met Met His Arg Leu Arg His Ser Arg Val Val Lys Leu Leu Gly
65              70                  75              80

Ile Ile Ile Glu Glu Gly Asn Tyr Ser Leu Val Met Glu Tyr Met Glu
             85              90                    95

Lys Gly Asn Leu Met His Val Leu Lys Thr Gln Ile Asp Val Pro Leu
             100             105                   110

Ser Leu Lys Gly Arg Ile Ile Val Glu Ala Ile Glu Gly Met Cys Tyr
         115             120                   125

Leu His Asp Lys Gly Val Ile His Lys Asp Leu Lys Pro Glu Asn Ile
    130             135                   140

Leu Val Asp Arg Asp Phe His Ile Lys Ile Ala Asp Leu Gly Val Ala
145             150                   155                   160

Ser Phe Lys Thr Trp Ser Lys Leu Thr Lys Glu Lys Asp Asn Lys Gln
             165                   170                   175

Lys Glu Val Ser Ser Thr Thr Lys Lys Asn Asn Gly Gly Thr Leu Tyr
         180                   185                   190

Tyr Met Ala Pro Glu His Leu Asn Asp Ile Asn Ala Lys Pro Thr Glu
         195                   200                   205

Lys Ser Asp Val Tyr Ser Phe Gly Ile Val Leu Trp Ala Ile Phe Ala
    210                   215                   220

Lys Lys Glu Pro Tyr Glu Asn Val Ile Cys Thr Glu Gln Phe Val Ile
225                   230                   235                   240

Cys Ile Lys Ser Gly Asn Arg Pro Asn Val Glu Glu Ile Leu Glu Tyr
             245                   250                   255

Cys Pro Arg Glu Ile Ile Ser Leu Met Glu Arg Cys Trp Gln Ala Ile
         260                   265                   270

Pro Glu Asp Arg Pro Thr Phe Leu Gly Ile Glu Glu Glu Phe Arg Pro
         275                   280                   285

Phe Tyr Leu Ser His Phe Glu Glu Tyr Val Glu Glu Asp Val Ala Ser
    290                   295                   300

Leu Lys Lys Glu Tyr Pro Asp Gln Ser Pro Val Leu Gln Arg Met Phe
305                   310                   315                   320

Ser Leu Gln His Asp Cys Val Pro Leu Pro Pro Ser Arg Ser Asn Ser
             325                   330                   335

Glu Gln Pro Gly Ser Leu His Ser Ser Gln Gly Leu Gln Met Gly Pro
             340                   345                   350
```

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```
Val Glu Glu Ser Trp Phe Ser Ser Ser Pro Glu Tyr Pro Gln Asp Glu
    355                 360             365

Asn Asp Arg Ser Val Gln Ala Lys Leu Gln Glu Glu Ala Ser Tyr His
370                 ,   375             380

Ala Phe Gly Ile Phe Ala Glu Lys Gln Thr Lys Pro Gln Pro Arg Gln
385                 390             395             400

Asn Glu Ala Tyr Asn Arg Glu Glu Glu Arg Lys Arg Arg Val Ser His
                405             410                 415

Asp Pro Phe Ala Gln Gln Arg Ala Arg Glu Asn Ile Lys Ser Ala Gly
                420             425             430

Ala Arg Gly His Ser Asp Pro Ser Thr Thr Ser Arg Gly Ile Ala Val
        435             440             445

Gln Gln Leu Ser Trp Pro Ala Thr Gln Thr Val Trp Asn Asn Gly Leu
    450             455             460

Tyr Asn Gln His Gly Phe Gly Thr Thr Gly Thr Gly Val Trp Tyr Pro
465             470             475             480

Pro Asn Leu Ser Gln Met Tyr Ser Thr Tyr Lys Thr Pro Val Pro Glu
            485             490             495

Thr Asn Ile Pro Gly Ser Thr Pro Thr Met Pro Tyr Phe Ser Gly Pro
            500             505             510

Val Ala Asp Asp Leu Ile Lys Tyr Thr Ile Phe Asn Ser Ser Gly Ile
        515             520             525

Gln Ile Gly Asn His Asn Tyr Met Asp Val Gly Leu Asn Ser Gln Pro
    530             535             540

Pro Asn Asn Thr Cys Lys Glu Glu Ser Thr Ser Arg His Gln Ala Ile
545             550             555             560

Phe Asp Asn Thr Thr Ser Leu Thr Asp Glu His Leu Asn Pro Ile Arg
            565             570             575

Glu Asn Leu Gly Arg Gln Trp Lys Asn Cys Ala Arg Lys Leu Gly Phe
            580             585             590

Thr Glu Ser Gln Ile Asp Glu Ile Asp His Asp Tyr Glu Arg Asp Gly
    595             600             605

Leu Lys Glu Lys Val Tyr Gln Met Leu Gln Lys Trp Leu Met Arg Glu
    610             615             620

Gly Thr Lys Gly Ala Thr Val Gly Lys Leu Ala Gln Ala Leu His Gln
625             630             635             640

Cys Cys Arg Ile Asp Leu Leu Asn His Leu Ile Arg Ala Ser Gln Ser
            645             650             655
```

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 2137 base pairs
        (B) TYPE: nucleic acid

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
ATGCAACCAG ACATGTCCTT GAATGTCATT AAGATGAAAT CCAGTGACTT CCTGGAGAGT      60

GCAGAACTGG ACAGCGGAGG CTTCGGGAAG GTGTCTCTGT GTTTCCACAG AACCCAGGGA     120

CTCATGATCA TGAAAACAGT GTACAAGGGG CCCAACTGCA TTGAGCACAA CGAGGCCCTC     180

TTGGAGGAGG CGAAGATGAT GAACAGACTG AGACACAGCC GGGTGGTGAA GCTCCTGGGC     240

GTCATCATAG AGGAAGGGAA GTACTCCCTG GTGATGGAGT ACATGGAGAA GGGCAACCTG     300

ATGCACGTGC TGAAAGCCGA GATGAGTACT CCGCTTTCTG TAAAAGGAAG GATAATTTTG     360

GAAATCATTG AAGGAATGTG CTACTTACAT GGAAAAGGCG TGATACACAA GGACCTGAAG     420

CCTGAAAATA TCCTTGTTGA TAATGACTTC CACATTAAGA TCGCAGACCT CGGCCTTGCC     480

TCCTTTAAGA TGTGGAGCAA ACTGAATAAT GAAGAGCACA ATGAGCTGAG GGAAGTGGAC     540

GGCACCGCTA AGAAGAATGG CGGCACCCTC TACTACATGG CGCCCGAGCA CCTGAATGAC     600

GTCAACGCAA AGCCCACAGA GAAGTCGGAT GTGTACAGCT TTGCTGTAGT ACTCTGGGCG     660

ATATTTGCAA ATAAGGAGCC ATATGAAAAT GCTATCTGTG AGCAGCAGTT GATAATGTGC     720

ATAAAATCTG GAACAGGCCA GATGTGGAT GACATCACTG AGTACTGCCC AAGAGAAATT     780

ATCAGTCTCA TGAAGCTCTG CTGGGAAGCG AATCCGGAAG CTCGGCCGAC ATTTCCTGGC     840

ATTGAAGAAA AATTTAGGCC TTTTTATTTA AGTCAATTAG AAGAAAGTGT AGAAGAGGAC     900

GTGAAGAGTT TAAAGAAAGA GTATTCAAAC GAAAATGCAG TTGTGAAGAG AATGCAGTCT     960

CTTCAACTTG ATTGTGTGGC AGTACCTTCA AGCCGGTCAA ATTCAGCCAC AGAACAGGCT    1020

GGTTCACTGC ACAGTTCCCA GGGACTTGGG ATGGGTCCTG TGGAGGAGTC CTGGTTTGGT    1080

CCTTCCCTGG AGCACCCACA AGAAGAGAAT GAGCCCAGCC TGCAGAGTAA ACTCCAAGAC    1140

GAAGCCAACT ACCATCTTTA TGGCAGCCGC ATGGACAGGC AGACGAAACA GCAGCCCAGA    1200

CAGAATGTGG CTTACAACAG AGAGGAGGAA AGGAGACGCA GGGTCTCCCA TGACCCTTTT    1260

GCACAGCAAA GACCTTACGA GAATTTTCAG AATACAGAGG GAAAAGGCAC TGTTTATTCC    1320

AGTGCAGCCA GTCATGGTAA TGCAGTGCAC CAGCCATCAG GGCTCACCAG CCAACCTCAA    1380

GTACTGTATC AGAACAATGG ATTATATAGC TCACATGGCT TTGGAACAAG ACCACTGGAT    1440

CCAGGAACAG CAGGTCCCAG AGTTTGGTAC AGGCCAATTC CAAGTCATAT GCCTAGTCTG    1500

CATAATATCC CAGTGCCTGA GACCAACTAT CTAGGAAATT CTCCACCAT GCCATTCAGC    1560

TCCTTGCCAC CAACAGATGA ATCTATAAAA TATACCATAT ACAATAGTAC TGGCATTCAG    1620
```

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```
ATTGGAGCCT ACAATTATAT GGAGATTGGT GGGACGAGTT CATCACTACT AGACAGCACA    1680

AATACGAACT TCAAAGAAGA GCCAGCTGCT AAGTACCAAG CTATCTTTGA TAATACCACT    1740

AGTCTGACGG ATAAACACCT GGACCCAATC AGGGAAAATC TGGGAAAGCA CTGGAAAAAC    1800

TGTGCCCGTA AACTGGGCTT CACACAGTCT CAGATTGATG AAATTGACCA TGACTATGAG    1860

CGAGATGGAC TGAAAGAAAA GGTTTACCAG ATGCTCCAAA AGTGGGTGAT GAGGGAAGGC    1920

ATAAAGGGAG CCACGGTGGG GAAGCTGGCC CAGGCGCTCC ACCAGTGTTC CAGGATCGAC    1980

CTTCTGAGCA GCTTGATTTA CGTCAGCCAG AACTAACCCT GGATGGGCTA CGGCAGCTGA    2040

AGTGGACGCC TCACTTAGTG GATAACCCCA GAAAGTTGGC TGCCTCAGAG CATTCAGAAT    2100

TCTGTCCTCA CTGATAGGGG TTCTGTGTCT GCAGAAA                            2137
```

(2) INFORMATION FOR SEQ ID NO:17:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 709 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: not relevant
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Met Gln Pro Asp Met Ser Leu Asn Val Ile Lys Met Lys Ser Ser Asp
1               5                   10                  15

Phe Leu Glu Ser Ala Glu Leu Asp Ser Gly Gly Phe Gly Lys Val Ser
            20                  25                  30

Leu Cys Phe His Arg Thr Gln Gly Leu Met Ile Met Lys Thr Val Tyr
        35                  40                  45

Lys Gly Pro Asn Cys Ile Glu His Asn Glu Ala Leu Leu Glu Glu Ala
    50                  55                  60

Lys Met Met Asn Arg Leu Arg His Ser Arg Val Val Lys Leu Leu Gly
65                  70                  75                  80

Val Ile Ile Glu Glu Gly Lys Tyr Ser Leu Val Met Glu Tyr Met Glu
                85                  90                  95

Lys Gly Asn Leu Met His Val Leu Lys Ala Glu Met Ser Thr Pro Leu
            100                 105                 110

Ser Val Lys Gly Arg Ile Ile Leu Glu Ile Ile Glu Gly Met Cys Tyr
        115                 120                 125

Leu His Gly Lys Gly Val Ile His Lys Asp Leu Lys Pro Glu Asn Ile
    130                 135                 140

Leu Val Asp Asn Asp Phe His Ile Lys Ile Ala Asp Leu Gly Leu Ala
145                 150                 155                 160
```

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Ser Phe Lys Met Trp Ser Lys Leu Asn Asn Glu Glu His Asn Glu Leu
                  165   170   175

Arg Glu Val Asp Gly Thr Ala Lys Lys Asn Gly Gly Thr Leu Tyr Tyr
            180   185   190

Met Ala Pro Glu His Leu Asn Asp Val Asn Ala Lys Pro Thr Glu Lys
        195   200   205

Ser Asp Val Tyr Ser Phe Ala Val Val Leu Trp Ala Ile Phe Ala Asn
        210   215   220

Lys Glu Pro Tyr Glu Asn Ala Ile Cys Glu Gln Gln Leu Ile Met Cys
225   230   235   240

Ile Lys Ser Gly Asn Arg Pro Asp Val Asp Asp Ile Thr Glu Tyr Cys
                245   250   255

Pro Arg Glu Ile Ile Ser Leu Met Lys Leu Cys Trp Glu Ala Asn Pro
            260   265   270

Glu Ala Arg Pro Thr Phe Pro Gly Ile Glu Glu Lys Phe Arg Pro Phe
        275   280   285

Tyr Leu Ser Gln Leu Glu Glu Ser Val Glu Glu Asp Val Lys Ser Leu
    290   295   300

Lys Lys Glu Tyr Ser Asn Glu Asn Ala Val Val Lys Arg Met Gln Ser
305   310   315   320

Leu Gln Leu Asp Cys Val Ala Val Pro Ser Ser Arg Ser Asn Ser Ala
                325   330   335

Thr Glu Gln Ala Gly Ser Leu His Ser Ser Gln Gly Leu Gly Met Gly
            340   345   350

Pro Val Glu Glu Ser Trp Phe Gly Pro Ser Leu Glu His Pro Gln Glu
        355   360   365

Glu Asn Glu Pro Ser Leu Gln Ser Lys Leu Gln Asp Glu Ala Asn Tyr
    370   375   380

His Leu Tyr Gly Ser Arg Met Asp Arg Gln Thr Lys Gln Gln Pro Arg
385   390   395   400

Gln Asn Val Ala Tyr Asn Arg Glu Glu Glu Arg Arg Arg Arg Val Ser
            405   410   415

His Asp Pro Phe Ala Gln Gln Arg Pro Tyr Glu Asn Phe Gln Asn Thr
        420   425   430

Glu Gly Lys Gly Thr Val Tyr Ser Ser Ala Ala Ser His Gly Asn Ala
        435   440   445

Val His Gln Pro Ser Gly Leu Thr Ser Gln Pro Gln Val Leu Tyr Gln
    450   455   460

Asn Asn Gly Leu Tyr Ser Ser His Gly Phe Gly Thr Arg Pro Leu Asp
465   470   ·75   480

Pro Gly Thr Ala Gly Pro Arg Val Trp Tyr Arg Pro Ile Pro Ser His
                485   490   495

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```
Met Pro Ser Leu His Asn Ile Pro Val Pro Glu Thr Asn Tyr Leu Gly
         500                 505                 510

Asn Ser Pro Thr Met Pro Phe Ser Ser Leu Pro Pro Thr Asp Glu Ser
         515                 520                 525

Ile Lys Tyr Thr Ile Tyr Asn Ser Thr Gly Ile Gln Ile Gly Ala Tyr
         530                 535                 540

Asn Tyr Met Glu Ile Gly Gly Thr Ser Ser Ser Leu Leu Asp Ser Thr
545                 550                 555                 560

Asn Thr Asn Phe Lys Glu Glu Pro Ala Ala Lys Tyr Gln Ala Ile Phe
                 565                 570                 575

Asp Asn Thr Thr Ser Leu Thr Asp Lys His Leu Asp Pro Ile Arg Glu
             580                 585                 590

Asn Leu Gly Lys His Trp Lys Asn Cys Ala Arg Lys Leu Gly Phe Thr
         595                 600                 605

Gln Ser Gln Ile Asp Glu Ile Asp His Asp Tyr Glu Arg Asp Gly Leu
         610                 615                 620

Lys Glu Lys Val Tyr Gln Met Leu Gln Lys Trp Val Met Arg Glu Gly
625                 630                 635                 640

Ile Lys Gly Ala Thr Val Gly Lys Leu Ala Gln Ala Leu His Gln Cys
             645                 650                 655

Ser Arg Ile Asp Leu Leu Ser Ser Leu Ile Tyr Val Ser Gln Asn Pro
             660                 665                 670

Trp Met Gly Tyr Gly Ser Ser Gly Arg Leu Thr Trp Ile Thr Pro Glu
         675                 680                 685

Ser Trp Leu Pro Gln Ser Ile Gln Asn Ser Val Leu Thr Asp Arg Gly
         690                 695                 700

Ser Val Ser Ala Glu
705
```

(2) INFORMATION FOR SEQ ID NO:18:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 38 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Ser Arg His Gln Ala Ile Phe Asp Asn Thr Thr Ser Leu Thr Asp Glu
1                 5                 10                 15
```

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```
His Leu Asn Pro Ile Arg Glu Asn Leu Gly Arg Gln Trp Lys Asn Cys
        20                  25                  30

Ala Arg Lys Leu Gly Phe
        35
```

(2) INFORMATION FOR SEQ ID NO:19:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 38 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Ala Lys Tyr Gln Ala Ile Phe Asp Asn Thr Thr Ser Leu Thr Asp Lys
1                5                  10                  15

His Leu Asp Pro Ile Arg Glu Asn Leu Gly Lys His Trp Lys Asn Cys
        20                  25                  30

Ala Arg Lys Leu Gly Phe
        35
```

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Ser Lys Tyr Ile Pro Arg Ile Ala Glu Asp Met Thr Ile Gln Glu Ala
1                5                  10                  15

Lys Lys Phe Ala Arg Glu Asn Asn Ile
        20                  25
```

(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met Thr Leu Ser Gln Val
1   5   10   15

Lys Gly Phe Val Arg Lys Asn Gly Val
            20   25

(2) INFORMATION FOR SEQ ID NO:22:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 40 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: not relevant
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Lys Trp Glu Asp Ser Ala His Pro Gln Arg Pro Asp Asn Ala Asp Leu
1   5   10   15

Ala Ile Leu Tyr Ala Val Val Asp Gly Val Pro Pro Ala Arg Trp Lys
            20   25   30

Glu Phe Met Arg Phe Met Gly Leu
        35   40

(2) INFORMATION FOR SEQ ID NO:23:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 41 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: not relevant
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp Thr Asp Asp
1   5   10   15

Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro Leu Arg Trp
            20   25   30

Lys Glu Phe Val Arg Arg Leu Gly Leu
        35   40

(2) INFORMATION FOR SEQ ID NO:24:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 42 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: not relevant
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Thr Glu Ser Gln Ile Asp Glu Ile Asp His Asp Tyr Glu Arg Asp Gly
1   5   10   15

Leu Lys Glu Lys Val Tyr Gln Met Leu Gln Lys Trp Leu Met Arg Glu
            20   25   30

Gly Thr Lys Gly Ala Thr Val Gly Lys Leu
        35   40

(2) INFORMATION FOR SEQ ID NO:25:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Thr Gln Ser Gln Ile Asp Glu Ile Asp His Asp Tyr Glu Arg Asp Gly
1   5   10   15

Leu Lys Glu Lys Val Tyr Gln Met Leu Gln Lys Trp Val Met Arg Glu
            20   25   30

Gly Ile Lys Gly Ala Thr Val Gly Lys Leu
        35   40

(2) INFORMATION FOR SEQ ID NO:26:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 40 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Lys Glu Gly Lys Ile Asp Glu Ile Met His Asp Ser Ile Gln Asp Thr
1   5   10   15

Ala Glu Gln Lys Val Gln Leu Leu Leu Cys Trp Tyr Gln Ser His Gly
            20   25   30

Lys Ser Asp Ala Tyr Gln Asp Leu
        35   40

(2) INFORMATION FOR SEQ ID NO:27:

    (i) SEQUENCE CHARACTERISTICS:

```
        (A) LENGTH: 40 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: protein
```

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
 Asn Glu Ala Lys Ile Asp Glu Ile Lys Asn Asp Asn Val Gln Asp Thr
 1             5                 10                 15

 Ala Glu Gln Lys Val Gln Leu Leu Arg Asn Trp His Gln Leu His Gly
            20                 25                 30

 Lys Lys Glu Ala Tyr Asp Thr Leu
        35                 40
```

(2) INFORMATION FOR SEQ ID NO:28:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 41 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: protein
```

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
 Ser Glu His Glu Ile Glu Arg Leu Glu Met Gln Asn Gly Arg Cys Leu
 1             5                 10                 15

 Arg Glu Ala Gln Tyr Ser Met Leu Glu Ala Trp Arg Arg Arg Thr Pro
            20                 25                 30

 Arg His Glu Asp Thr Leu Glu Val Val
        35                 40
```

(2) INFORMATION FOR SEQ ID NO:29:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 41 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: protein
```

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
 Ser Asp His Glu Ile Asp Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu
 1             5                 10                 15
```

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```
Arg Glu Ala Gln Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro
          20                  25              30

Arg Arg Glu Ala Thr Leu Glu Leu Leu
       35                    40
```

(2) INFORMATION FOR SEQ ID NO:30:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 23 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: not relevant
  (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: protein

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```
Ala Gln Ala Leu His Gln Cys Cys Arg Ile Asp Leu Leu Asn His Leu
1               5                   10                  15

Ile Leu Arg Ala Ser Gln Ser
              20
```

(2) INFORMATION FOR SEQ ID NO:31:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 22 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: not relevant
  (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: protein

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```
Ala Gln Ala Leu His Gln Cys Ser Arg Ile Asp Leu Leu Ser Ser Leu
1               5                   10                  15

Ile Tyr Val Ser Gln Asn
              20
```

(2) INFORMATION FOR SEQ ID NO:32:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 41 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: not relevant
  (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: protein

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

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```
Ile Lys Gly Leu Lys Lys Ala Glu Cys Arg Arg Thr Leu Asp Lys Phe
1               5               10                      15

Gln Asp Met Val Gln Lys Asp Leu Gly Lys Ser Thr Pro Asp Thr Gly
            20                      25                  30

Asn Glu Asn Glu Gly Gln Cys Leu Glu
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:33:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 41 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu Ala Glu Lys Ile
1               5               10                      15

Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser Asp Ser Glu Asn Ser Asn
            20                      25                  30

Phe Arg Asn Glu Ile Gln Ser Leu Val
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:34:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
Gly Leu Val Leu Ser Lys Met Asn Leu Ala Gly Cys Leu Glu Asn Ile
1               5               10                      15

Leu Glu Ala Leu Arg Asn Pro Ala Pro Ser Ser Thr Thr Arg Leu Pro
            20                      25                  30

Arg
```

(2) INFORMATION FOR SEQ ID NO:35:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant

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    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly Cys Leu Glu Asp Ile
1   5   10   15

Glu Glu Ala Leu Cys Cys Gly Pro Ala Ala Leu Pro Pro Ala Pro Ser
            20   25   30

Leu Leu Arg
        35

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Claims

We claim:

1. An isolated receptor interacting protein
(RIP), comprising a death domain in its carboxy terminal
region and a kinase domain in its amino terminal region,
said RIP being capable of interacting with a Fas/APO-1
intracellular domain, and being capable of promoting
apoptosis in a eukaryotic cell.

2. The RIP of claim 1, said RIP being a human
protein.

3. The RIP of claim 2, said RIP comprising an
amino acid sequence consisting of (SEQ ID NO:17).

4. The RIP of claim 1, said RIP being a murine
protein.

5. The RIP of claim 4, said RIP comprising an
amino acid sequence consisting of (SEQ ID NO:15).

6. An isolated polypeptide comprising the
death domain of an RIP.

7. An isolated polypeptide comprising the
kinase domain of an RIP.

8. An isolated DNA molecule comprising a
nucleotide sequence encoding an RIP, said RIP comprising
a death domain in its carboxy terminal region and a
kinase domain in its amino terminal region, said RIP
being capable of interacting with a Fas intracellular
domain, and said RIP being capable of promoting apoptosis
in a eukaryotic cell.

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9. The isolated DNA molecule of claim 8, wherein said nucleotide sequence encodes a human RIP.

10. The isolated DNA molecule of claim 8, wherein said nucleotide sequence encodes a murine RIP.

5   11. An isolated DNA molecule comprising a nucleotide sequence encoding an RIP death domain.

12. An isolated DNA molecule comprising a nucleotide sequence encoding an RIP kinase domain.

13. A transformed host cell comprising a
10   recombinant DNA molecule encoding an RIP.

14. The transformed host cell of claim 13, said transformed host cell being a cultured cell of a metazoan animal.

15. The transformed host cell of claim 13,
15   said transformed host cell being prokaryotic.

16. A polyclonal antibody preparation obtained by immunizing an animal with an isolated protein comprising an RIP death domain, said polyclonal antibody preparation having binding activity directed to the RIP
20   intracellular domain.

17. The polyclonal antibody preparation of claim 16, wherein said binding activity directed to the RIP death domain is substantially greater than the binding activity directed to any member of the NGFR/TNFR
25   protein family.

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18. A monoclonal antibody that reacts with an
RIP death domain.

19. The monoclonal antibody of claim 18, whose
binding directed to the RIP intracellular domain is
5   substantially greater than the binding activity directed
to any member of the NGFR/TNFR protein family.

20. A cellular screening method for
identifying chemical compounds capable of inhibiting RIP-
dependent promotion of apoptosis, said method comprising
10   the steps of:
          (a) expressing recombinant RIP in a transformed
host cell;
          (b) contacting said transformed host cell with
an inhibitor candidate compound;
15   (c) detecting morphological or biochemical
indicia of apoptosis in said transformed host cell at an
appropriate time interval following expression of
recombinant RIP; and
          (d) comparing the detection in step (c) with
20   the corresponding detections from one or more appropriate
experimental controls.

21. The method of claim 20, wherein said
recombinant RIP is expressed under the control of an
inducible promoter.

25   22. The method of claim 20, wherein step (b)
precedes step (a).

23. A cell-free screening method for
identifying chemical compounds capable of inhibiting RIP-
dependent promotion of apoptosis, said method comprising
30   the steps of:

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        (a) contacting, in the presence of an inhibitor candidate compound, a protein comprising the death domain of RIP with an RIP interaction partner;

        (b) detecting a specific interaction, in the
5   presence of an inhibitor candidate compound, between said protein comprising the death domain of RIP and said RIP interaction partner;

        (c) contacting, in the absence of said inhibitor candidate compound, said protein comprising the
10   death domain of RIP with said RIP interaction partner;

        (d) detecting a specific interaction, in the absence of said inhibitor candidate compound, between said protein comprising the death domain of RIP and said RIP interaction partner; and

15   (e) comparing the detection in step (b) and the detection in step (d).

        24.   The method of claim 23, wherein said RIP interaction partner is Fas/APO-1.

        25.   The method of claim 23, wherein said RIP
20   interaction partner is inserted into a biological membrane.

        26.   A cellular screening method for identifying chemical compounds capable of inducing RIP expression and apoptosis resulting therefrom, said method
25   comprising the steps of:

        (a) culturing a eukaryotic cell;

        (b) contacting said eukaryotic cell in culture with a RIP-inducing compound candidate;

        (c) detecting morphological or biochemical
30   indicia of apoptosis in said transformed host cell at an appropriate time interval following expression of recombinant RIP; and

(d) comparing the detection in step (c) with the corresponding detections from one or more appropriate experimental controls.

5   27.   A method of producing, for introduction into a patient undergoing gene therapy, cells that can be induced to undergo apoptosis upon administration of an exogenous agent to said patient.
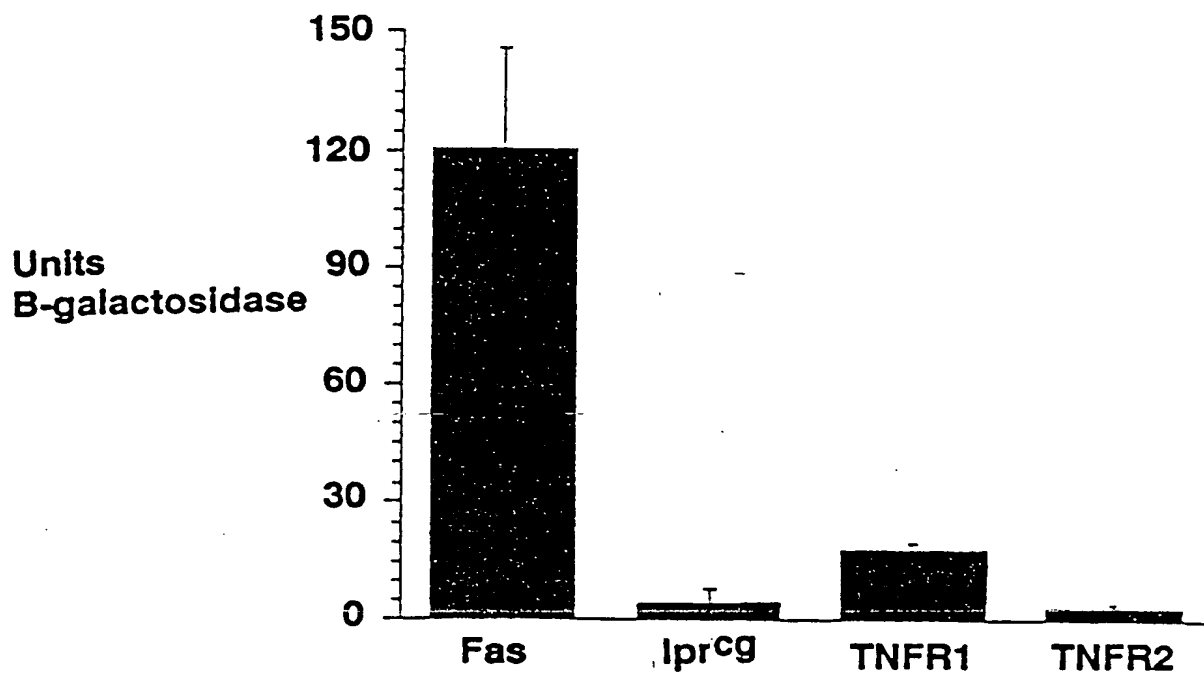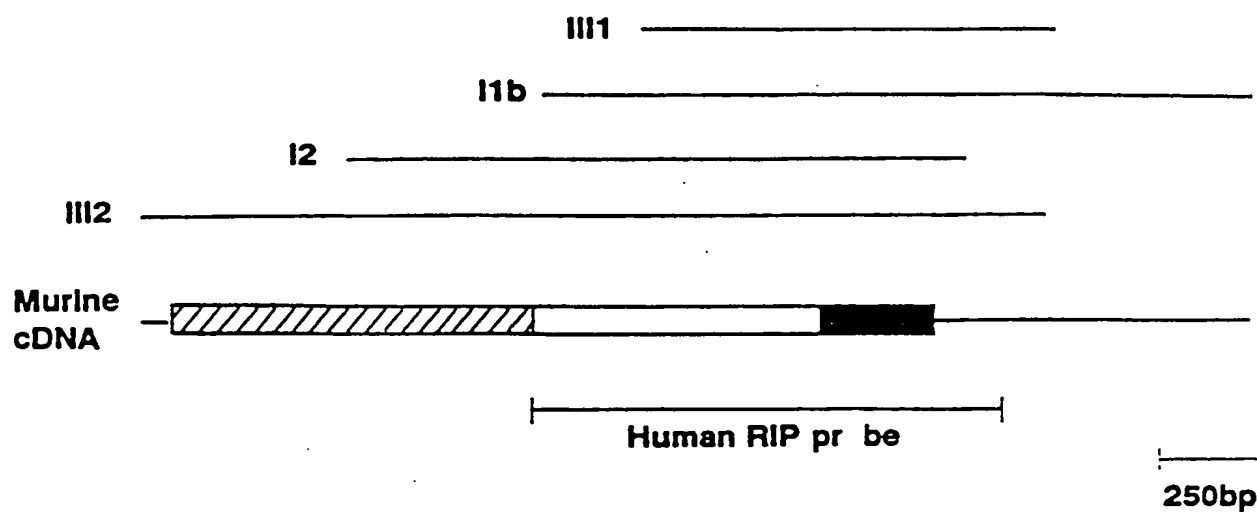
FIG. 1

FIG. 2

(SEQ ID NO:15)

```
1    M Q P D M S L D N I K M A S S D L L E K T D L D S G G F G K V S L C Y H R S H G

41   F V I L K K V Y T G P N R A E Y N E V L L E E G K M M H R L R H S R V V K L L G

81   I I I E E G N Y S L V M E Y M E K G N L M H V L K T Q I D V P L S L K G R I I V

121  E A I E G M C Y L H D K G V I H K D L K P E N I L V D R D F H I K I A D L G V A

161  S F K T W S K L T K E K D N K Q K E V S S T T K K N N G G T L Y Y M A P E H L N

201  D I N A K P T E K S D V Y S F G I V L W A I F A K K E P Y E N V I C T E Q F V I

241  C I K S G N R P N V E E I L E Y C P R E I I S L M E R C W Q A I P E D R P T F L

281  G I E E E F R P F Y L S H F E E Y V E E D V A S L K K E Y P D Q S P V L Q R M F
  1                                          - - K - - - - - - S N E N A - V K R - Q

321  S L Q H D C V P L P P S R S N S . . E Q P G S L H S S Q G L Q M G P V E E S W F
  21 - - . - L - - - A V - S - - - - - A T - - - - - - - - - - - G - - - - - - - -

359  S S S P E Y P Q D E N D R S V Q A K L Q E E A S Y H A F G I F A E K Q T K P Q P
  61 A P - L - H - - E - - E P - L - S - - - D - - N - - L Y - S R M D R - - - Q - -

399  R Q N E A Y N R E E E R K R R V S H D P F A Q Q R A R E N I K S A G A R G H S D
  101 - - - V - - - - - - - - R - - - - - - - - - - P Y - - F Q N T E G K - T V Y

439  P S T T S R G I A V Q Q . . . L S W P A T Q T V W N N G L Y N Q H G F G T . . .
  141 S - A A - H - N - - H - P S G - T S Q P Q V L Y Q - - - - - S S - - - - - R P L

473  . . . . T G T G V W Y P P N L S Q M Y S T Y K T P V P E T N I P G S T P T M P Y
  181 D P G T A - P R - - - R - I P - H - P - L H N I - - - - - Y L - N S - - - - P

509  F S G P V A D D L I K Y T I F N S S G I Q I G N H N Y M D V G L N S . . . . Q P
  221 S - L - P T - E S - - - - Y - - T - - - - - A Y - - - E I - G T - S S L L D S

545  P N N T C K E E S T S R H Q A I F D N T T S L T D E H L N P I R E N L G R Q W K
  261 T - T N F - - - P A A K Y - - - - - - - - - - - - K - - D - - - - - - - K H - -

585  N C A R K L G F T E S Q I D E I D H D Y E R D G L K E K V Y Q M L Q K W L M R E
  301 - - - - - - - - Q - - - - - - - - - - - - - - - - - - - - - - - - - - V - - -

625  G T K G A T V G K L A Q A L H Q C C R I D L L N H L I R A S Q S
  341 - I - - - - - - - - - - - - S - - - - - S S - - Y V - - N
```

FIG. 3

Consensus

		k													K				R	

Mouse RIP   555   S R H Q A I F D N T T S L T D N - - - - - L N P I R E N L G R - Q V K N C A R K L G F   (SEQ ID NO:18)
Human RIP   271   A K Y Q A I F D N T T S L T D K - - - - - L D P I R E N L G K - H V K N C A R K L G F   (SEQ ID NO:19)
Mouse FAS   201   S K Y I P R I A E D M T I Q G A - - - - - - - - - - T Q F V R E N N I   (SEQ ID NO:20)
Human FAS   214   S K Y I T T I A G V M T L S Q V - - - - - - - - - - - - - K G F V R K N G V   (SEQ ID NO:21)
Mouse p55 TNFR   313   - K W E D S A H - P Q R P D N A D L A I L Y A V V D G - V P P A R W K E F M R R L G L   (SEQ ID NO:22)
Human p55 TNFR   312   - K W E D S A H K P Q S L P T D D P A T L Y A V V E N V P P L R W K E F V R R L G L   (SEQ ID NO:23)

Consensus

		d													W				R	

Mouse RIP   593   T E S Q I D E I D H D Y E R D G Y Q M L K E L Q W L Q K C V Y Q R E G T K G A T V G K L   (SEQ ID NO:24)
Human RIP   309   T Q S Q I D E I D D Y E R D G I Q M L K E V Q Y Q K V - Q R E G G I K A T V G K L   (SEQ ID NO:25)
Mouse FAS   226   K E C K I D E I N H D S I Q M L Q K V - Q K L S H O D A Y Q D L   (SEQ ID NO:26)
Human FAS   239   N E A K I D E I K N D N V Q R N A E Q K V - K E A Y D T L   (SEQ ID NO:27)
Mouse p55 TNFR   352   S E H E I R L E M Q N G R C L R E A Q Y S M L A T W R R P R H E D T L E V   (SEQ ID NO:28)
Human p55 TNFR   352   S D I E I D R L E L Q N G R C L R E A Q Y S M L A T W R R R T P R R E A T L E L L   (SEQ ID NO:29)

Consensus

		L																	R	

Mouse RIP   635   A Q A L H Q C - - - C R I D L - - - - L N H L I R A S Q S   (SEQ ID NO:30)
Human RIP   351   A Q A L H Q C - - - S R I D L - - - - I Y V S S L I Y V V Q R   (SEQ ID NO:31)
Mouse FAS   266   I K G L K K A E - C R R T L A - D K F Q D M V Q K D L G K S T P D T G N E E C Q C L L E   (SEQ ID NO:32)
Human FAS   279   I K D L K K A N L C T L A E K I Q T I L E D S W N Q R N F R N E I Q S L V   (SEQ ID NO:33)
Mouse p55 TNFR   393   G L V L S K M N L - A G C L E N I L E A L R N P A P - S S T T R L P R R   (SEQ ID NO:34)
Human p55 TNFR   393   G R V R D M D L L - L G C L E D I E E A L C G P A A L P P A P S L L R   (SEQ ID NO:35)

B

A

FIG. 4

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :Please See Extra Sheet.
US CL :435/6, 7.1, 69.1, 71.2, 240.2; 530/350, 388.15; 514/44; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/6, 7.1, 69.1, 71.2, 240.2; 530/350, 388.15; 514/44; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

. Sequence Searches - MasPar; APS and STN (Medline and CaPlus). Search Terms used: Programmed Cell Death; PCD and DNA; Apoptosis and DNA and protein and cell death; etc.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 74, issued 10 September 1993, Tartaglia et al., " A Novel Domain within the 55kd TNF Receptor Signals Cell Death". Pages 845-850. Especially see page 846, fig. 1, and page 847-48, Table 1 and fig. 3.	1-7
Y	Nature. Vol. 372, Issued 22/29 December 1994, Hubbard et al., " Crystal Structure of the Tyrosine Kinase Domain of the Human Insulin Receptor". See abstract and sequence alignment in fig.3.	1-15

X Further documents are listed in the continuation of Box C.　　☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 JUNE 1996	15 JUL 1996

| Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No.　(703) 305-3230 | Authorized officer
TEKCHAND SAIDHA
Telephone No.　(703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

	C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY. Vol. 270, No. 1, issued 06 January 1995, Boldin et al., "Self-association of the 'Death Domains' of the p55 Tumor Necrosis Factor (TNF) Receptor and Fas/APO1 Prompts Signaling for TNF and Fas/APO1 Effects. Especially see Table 1.	1-15
X --- Y	US 5,360,893 A (G.P. Owens et al.) 01 November 1994, columns 27-28, claims 1 & 2; column 2, lines 5-44.	11 -------- 20-27
Y	Science, Vol. 245, issued 21 July 1989, Trauth et al., "Monoclonal Antibody-Mediated Tumor Regression by Induction of Apoptosis", pages 301-305.	16-19
Y, P	US 5,500,432 A (K.C. NICOLAOU et al.) 19 March 1996. See columns 10-12, claims 1-4.	20-26
X, P	Cell, Vol. 81, issued 19 May 1995, Stanger et al., "RIP: A Novel Protein containing a Death Domain that interacts with Fas/APO-1 (CD95) in yeast and causes Cell Death.	1-27

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/68; G01N 33/35; C12P 21/04, 21/06; C12N 5/00; C07K 1/00, 16/00; A01N 43/04; C07H 21/04